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**Low Water Activity-Induced  
Inactivation of *Escherichia coli* -  
Kinetics, Processes and Applications**

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***by***

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## **Declaration and Copyright Statement**

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A handwritten signature in black ink, appearing to read 'O. McQuestin', with a long, sweeping horizontal stroke extending to the right.

Olivia McQuestin

March, 2006

## Abstract

Serious illness caused by *Escherichia coli* and associated with the consumption of foods characterised by low water activity (e.g. uncooked, comminuted fermented meat) has clearly demonstrated the ability of this microbe to survive adverse environments. The consequences of such disease outbreaks have included the loss of life, serious and sometimes long-term illness in other victims, and obviously a detrimental impact on the food industry that is implicated. The prevention of further outbreaks relies upon an improved understanding of the capacity of *E. coli* to survive inimical conditions. This thesis describes the kinetics of low water activity-induced inactivation of *E. coli*, including consideration of the combined effects of low pH, and examines the cell death processes involved. In addition, this study attempts to define whether cells that exhibit enhanced survival in conditions of low water activity do so by inducing a specific stress response.

Materials and methods commonly used to analyse bacterial viability and injury were initially evaluated to enable a more accurate description of *E. coli* inactivation in response to osmotic stress. The composition of the medium was observed to strongly influence the inactivation of *E. coli*. Traditional, culture-based methods were found to overstate the level of injury in low water activity-treated populations of *E. coli* and a modified procedure was employed subsequently. Methods were also developed to improve the level of reproducibility between experiments.

Initial studies using culture-based methods demonstrated that the inactivation of *E. coli* in response to low water activity consisted of three distinct phases of inactivation. The ability to induce the final, rapid phase of inactivation would be of considerable benefit during food manufacture processes to better reduce pathogenic

loads. Because the use of low water activity as a preservation method in food manufacture is often in combination with acid stress, the kinetics of inactivation of *E. coli* in response to low water activity and low pH were also investigated. Experiments showed that the order in which these non-thermal stresses were applied influenced the inactivation of *E. coli*. That is, cells were more sensitive to osmotic stress when first exposed to low pH conditions. These findings are of relevance to food manufacturing processes that use multiple stresses to ensure microbiological safety. The level of injury in osmotic or acid treated *E. coli* populations suggested that the processes responsible for the effect of these stresses were different.

The above knowledge was used to develop a broth-based model that mimicked the rates of inactivation of *E. coli* observed in uncooked, comminuted fermented meat products. This system allowed for the systematic generation of a large amount of data to define the response of *E. coli* to specific conditions without the limitations of in-product trials. The data generated in this work were incorporated into a predictive model that has since been used by food manufacturers and regulators to assess the ability of uncooked, comminuted fermented meat manufacturing processes to inactivate *E. coli*. In addition, this study highlighted the need to confirm patterns of microbial responses derived from broth-based systems with that from in-product trials. Although water activity was shown to influence the rate of inactivation of *E. coli* using the broth-based system, comparisons with in-product trials reported in the literature suggested that this was not the case in the actual food product.

Having characterised the kinetics of low water activity-induced inactivation of *E. coli*, subsequent work attempted to develop knowledge relating to the processes



involved in that system. It was hypothesised that the mechanism of inactivation of *E. coli* in response to low water activity involves specific genetic modules shown to mediate the death of bacterial cells in some situations. Using *E. coli* mutants deleted for one of these systems (*mazEF*), the involvement of this component could not be conclusively shown but nor could it be ruled out. The *mazEF* module might mediate the death of a proportion of *E. coli* cells that are killed immediately following exposure to some low water activity environments. The implications of this self-mediated cell death pathway on the present understanding of bacterial inactivation are discussed.

Finally, investigations aimed at identifying a specific stress response that enhances the survival of *E. coli* in lethal water activity environments provided no direct evidence for such a response. Provision of the compatible solute betaine did not alter the survival characteristics of osmotically stressed *E. coli* and proteomic experiments indicated that four stress-related proteins do not form a specific response to lethal osmotic stress in *E. coli*. The use of proteomic techniques further provided a general overview of the physiology of *E. coli* that are able to survive osmotic challenges, which may be of considerable value when coupled with genomic studies that more comprehensively assess stress physiology in *E. coli*.

Overall, the work presented throughout this thesis develops the present understanding of the response of *E. coli* to inimical conditions relevant to the manufacture of uncooked, comminuted fermented meat products.

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**Commonly Used Abbreviations and Terms**

|                 |  |
|-----------------|--|
| 2-DE            | Two-dimensional electrophoresis  |
| $a_w$           | Water activity   |
| BHA-P           | Brain heart infusion agar with 0.1% sodium pyruvate                            |
| BHA-BS          | Brain heart infusion agar with 0.15% bile salts                                |
| BHI             | Brain heart infusion   |
| CMM             | Cooked meat medium   |
| EHEC            | Enterohaemorrhagic <i>Escherichia coli</i>                                     |
| FRT             | Flp recognition target   |
| FLS             | First lethal stress  |
| HACCP           | Hazard analysis and critical control point                                     |
| IEF             | Isoelectric focusing   |
| IPG             | Immobilised pH gradient  |
| Km <sup>R</sup> | Kanamycin resistant  |
| LB              | Luria-Bertani broth  |
| MALDI-TOF (MS)  | Matrix-assisted laser desorption ionisation-time of flight (mass spectrometry) |
| MM              | Minimal minerals medium with 0.1% D-glucose                                    |
| MM-B            | Minimal minerals medium containing 0.1% D-glucose and 2 mM betaine             |
| Mowse           | Molecular weight search  |
| MSE             | Mean square error  |
| NB              | Nutrient broth   |
| NCBI            | National Center for Biotechnology Information                                  |
| NM              | Non-motile   |
| P1, P2 and P3   | Phase of inactivation (first, second and third, respectively)                  |

|                           |  |
|---------------------------|--|
| PCD                       | Programmed cell death                                  |
| ppGpp                     | Guanosine-3',5'-bispyrophosphate                       |
| PRMS01                    | Predictive model described in Ross and Shadbolt (2001) |
| PRMS05.a <sub>w</sub> /pH | Predictive model developed in the current work         |
| RT                        | Room temperature                                       |
| SLS                       | Second lethal stress                                   |
| TA                        | Toxin-antitoxin  |
| TCA                       | Tricarboxylic acid                                     |
| TGS                       | Tris glycine sodium dodecyl sulphate                   |
| TSA                       | Tryptone soya agar                                     |
| TSB                       | Tryptone soya broth                                    |
| UCFM                      | Uncooked, comminuted fermented meat                    |
| Vaspar                    | A 1:1 mixture of petroleum jelly and paraffin oil      |

## **Chapter 1**

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# **Introduction – The Effect of Low Water Activity on *Escherichia coli***

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## 1.1 PATHOGENIC *ESCHERICHIA COLI*

First described in 1885 by Dr. Theodor Escherich (Escherich, 1989), the Gram-negative *Escherichia coli* has since become an extensively studied bacterium of the family *Enterobacteriaceae*. *E. coli* constitutes part of the normal microbiota of the gut of humans and other large mammals and was initially considered to be a harmless commensal until a study clearly demonstrated the ability of some strains of *E. coli* to cause diarrhoea in humans (Bray, 1945 cited by Doyle and Padhye, 1989). Since that time, the pathogenicity of specific *E. coli* strains has been described and these pathogenic *E. coli* are now known to cause a variety of clinical illnesses.

Pathogenic *E. coli* cause two classes of infection; extraintestinal infections, including neonatal meningitis, septicaemia and urinary tract infections (Ørskov, 1984), and gastrointestinal infections, which range from mild diarrhoea to a number of potentially fatal syndromes. Diarrhoeagenic *E. coli* are the specific cause of gastrointestinal infections and were identified as the main cause of foodborne gastroenteritis in Australia, causing an estimated 565 000 cases annually (Hall *et al.*, 2005). Diarrhoeagenic *E. coli* have been differentiated into six distinct groups according to differences in serology, epidemiology and the subsequent clinical syndromes. These groups are enteropathogenic, enterotoxigenic, enteroinvasive, enteroaggregative, diffusely adherent and enterohaemorrhagic *E. coli* (EHEC) (Buchanan and Doyle, 1997).

The EHEC group consists of a number of serotypes that are characterised by their production of shiga-like toxin I and/or shiga-like toxin II, also known as verotoxin 1 and verotoxin 2 (Buchanan and Doyle, 1997). These toxins enter the bloodstream

and bind to receptors most prevalent on renal cells to directly cause disease. Haemolytic colitis is the primary infection caused by EHEC and presents as bloody diarrhoea with abdominal pain (Buchanan and Doyle, 1997). Complications of haemolytic colitis include haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura, which are potentially fatal diseases, particularly in children, the elderly and the immunocompromised. Importantly, EHEC is further characterised by its low infective dose; in some cases the consumption of less than 50 cells may cause infection (Tilden *et al.*, 1996).

## **1.2 ENTEROHAEMORRHAGIC *ESCHERICHIA COLI* AND MEAT**

EHEC are a significant concern to the meat industry as foods of bovine origin have been implicated as the predominant vehicle in disease outbreaks (Padhye and Doyle, 1992; Wachsmuth *et al.*, 1997). It was first recognised as a foodborne pathogen in 1982 when an outbreak of *E. coli* O157:H7 was associated with undercooked ground beef in the US (Riley, 1983). Since that time, outbreaks of EHEC infection associated with foods of bovine origin have included hamburger patties (Willshaw *et al.*, 1994), salami (CDC, 1995a), cold cooked meat (WHO, 1997), pasteurised milk (Upton and Coia, 1994) and raw milk (Martin *et al.*, 1986) and have for the most part been caused by *E. coli* O157:H7. In Australia the non-O157 serogroup *E. coli* O111:non-motile (NM) is the major EHEC of concern, having caused a serious outbreak of disease following the consumption of mettwurst (CDC, 1995b; Bettelheim, 2000).

Numerous studies have identified healthy cattle as the principle reservoir of EHEC (Wells *et al.*, 1991; Dargatz *et al.*, 1997; Smith *et al.*, 2001). Other reservoirs include sheep, goats, pigs, turkeys, chickens and deer (Keene *et al.*, 1997; Kudva *et*



*al.*, 1997; Heuvelink *et al.*, 1999). These animals harbour EHEC in their gastrointestinal tract and shed EHEC in their faeces. Although the muscle tissue of red meat animals does not contain EHEC (Gill, 1982), the muscle can be contaminated with this pathogen during primary production (i.e. at the abattoir). Faecal contamination of the external surfaces of hides, hooves and hair of red meat animals can result in the transfer of high levels of EHEC to muscle tissue, particularly during the process of hide removal (Newton *et al.*, 1978). Another major source of EHEC contamination occurs during evisceration, when EHEC in the gut microbiota can come into contact with the carcass surface (Jackson *et al.*, 1997) and, later during production, muscle tissue may become contaminated with EHEC that has previously contaminated processing equipment, such as meat grinders (Farrell *et al.*, 1998). Minimisation of the level of EHEC contamination of carcass surfaces is paramount to reducing the likelihood of associated illnesses. Although various methods, such as optimisation of housing conditions and controlling the animal's diet prior to slaughter (to reduce soiling of the hide) and the physical removal of visible faecal contamination from the carcass, might be used to reduce the incidence of EHEC contamination, contact of the muscle tissue with EHEC cannot be completely prevented in all cases (Vanderlinde *et al.*, 1998). As a result, the production of microbiologically safe meats further relies upon inhibition of the growth and/or survival of any pathogenic microorganisms that contaminate muscle tissue.

### **1.2.1 Parameters Controlling Growth and Survival**

A number of intrinsic and extrinsic parameters may limit the growth and/or cause the inactivation of EHEC in meat or meat products. Intrinsic parameters include properties that are inherent to the food itself such as pH, water activity ( $a_w$ ) and

nutrient content. Extrinsic parameters are those related to the storage environments that affect the food including temperature, relative humidity and gaseous atmosphere. For any environmental parameter, bacterial cells are able to grow only over a limited range, and exhibit an optimal level for growth. As that parameter deviates outside the required range for growth, bacteria become dormant and may even be inactivated. While the growth of a bacterial species in relation to a given parameter can be characterised by minimum, maximum and optimum (i.e. when the rate of growth is at its greatest) values, such estimates relate to systems in which all other growth-controlling factors have been optimised. Consequently, these values have limited application to foods that are most often characterised by a combination of environmental parameters that impede bacterial growth and survival (Troller, 1987) but they can provide a general indication of the capacity of bacteria to grow. The most influential factors on the growth and survival of pathogens, including EHEC, in meat environments are temperature, pH and  $a_w$ .

#### **1.2.1.1 Temperature**

*E. coli* growth can occur over a temperature range of approximately 40°C, although the exact optimum, minimum and maximum temperatures vary between strains. Buchanan and Bagi (1994) reported that the minimum temperature for growth of *E. coli* O157:H7 is approximately 8°C, whereas the optimum and maximum temperatures are around 37 and 46°C respectively (Salter *et al.*, 1998). The physiological basis for control of bacterial growth by temperature relates to its control on the rate of all physicochemical reactions (Herbert, 1989) and, therefore, it is not surprising that temperature is considered the most significant environmental factor controlling the multiplication of bacteria.

At temperatures above the optimum for growth of *E. coli* (approximately 40°C) increasing temperature causes growth rate reduction, then complete growth inhibition and ultimately results in bacterial inactivation. Pathogenic *E. coli* are not unusually heat resistant and have been reported to have a decimal reduction time (i.e. the time taken to reduce the population by greater than 90%) at 60°C of approximately 0.45 minutes in ground beef (Ahmed *et al.*, 1995). Because high temperatures have so many effects on bacterial cells it is difficult to define the exact lethal action of heat. Nevertheless, it is apparent that heat causes single strand breaks in DNA, increases the non-selective permeability of the cytoplasmic membrane and denatures proteins, thereby damaging cellular enzymes (reviewed by Gould, 1989a). It is likely that one, or a combination of these events, are responsible for the inactivation of the cell.

A freshly prepared meat carcass has a surface temperature of 25 – 30°C and deep muscle temperatures of 38 – 40°C (Eustace, 1981). This environment is favourable for the growth of EHEC. To minimise the growth of EHEC, and indeed any mesophilic microbe that may be present, the carcass is cooled within a specified time and usually to 7°C (Smith, 1985). The continued storage of meat at low temperatures will inhibit the growth of EHEC but will not cause inactivation. Instead, heating of the meat prior to consumption ensures the inactivation of EHEC.

#### **1.2.1.2 pH**

The pH of an environment is a measure of the concentrations of hydrogen and hydroxide ions. A low (or acidic) pH equates to a high concentration of hydrogen ions, which is often observed in fermented meat products. Acidic conditions increase the protonation of biologically active molecules and thereby cause changes in their structure and activity (Booth and Kroll, 1989). The pH range over which

growth of bacterial cells can occur tends to mimic the pH range where these molecules have optimum stability and activity. Thus, *E. coli* grows in the range of pH 4.0 – 10.0 (Desmarchelier and Grau, 1997; Presser *et al.*, 1998) and growth rates are maximal between pH 6.0 and 8.0 (Ingraham and Marr, 1996).

The inactivation of bacterial cells in response to low pH environments is generally held to involve the extensive protonation and, thereby, inhibition of an enzyme that is essential for survival of the cell (Booth and Kroll, 1989; Small *et al.*, 1994). The identification of such an enzyme, however, remains to be elucidated. Because acidification of foods is typically due to the presence of organic (or weak) acids, the undissociated species or its anion may additionally inhibit cellular processes and lead to inactivation in a manner that is independent of the acidification of the cytoplasm (Salmond *et al.*, 1984).

The pH of meat at the time of slaughter tends to be pH 7.0 - 7.2 and is reduced to pH 5.5 - 5.7 due to glycolysis (Gill and Newton, 1978). Although the exact pH is dependent on the carcass and muscle type, these values are typically within the range required for growth of EHEC. The pH of some meat products, such as salami, is deliberately reduced by the direct addition of an acidulant or due to the production of lactic acid by fermentative bacteria inoculated during processing. In these instances, the pH often becomes growth limiting and can cause the inactivation of EHEC.

The mechanisms of action involved in the temperature and pH preservation of meat and meat products is relatively well understood. In contrast, the processes involved in the growth inhibition and inactivation of EHEC in response to environmental  $a_w$

values are poorly defined. As the focus of the current study, this chapter now turns to a more comprehensive review of this topic.

### **1.3 THE EFFECT OF LOW WATER ACTIVITY ON *ESCHERICHIA COLI***

The term  $a_w$  refers to the availability of water in solution and is defined as the ratio of the water vapour pressure of the solution to the vapour pressure of pure water at the same temperature. Pure water has a  $a_w$  of 1.00, a 15% (weight per volume) NaCl solution has a  $a_w$  of approximately 0.90 and a saturated NaCl solution has a  $a_w$  of 0.75. In the context of food microbiology, the  $a_w$  of a food is an indication of the amount of water within that food that is available for microbes. It is not the total water content of the food because water-soluble salts, proteins and carbohydrates bind a proportion of that water.

#### **1.3.1 Growth Inhibition and Inactivation**

The bacterial cell membrane is essentially a barrier between the cytoplasm of bacteria and the surrounding environment. This barrier allows free passage of water whereas it is impermeable to solutes and consequently, upon changes in external osmolality, water moves across the lipid bilayer by osmosis. An increase in external osmolality, called an upshock, causes water to diffuse out of the cell and under extreme conditions results in plasmolysis, where the cell membrane shrinks away from the cell wall. In contrast, a reduction in external osmolality, or downshock, causes the influx of water into the cytoplasm and can lead to rupturing of the membrane. Under either of these conditions the bacterial cell is said to be stressed, however, the term 'osmotic stress' generally refers to exposure to an

environment of high osmolality, or low  $a_w$  (Csonka and Epstein, 1996) and will be used in this context throughout this thesis.

*E. coli*, like other bacteria, maintains an internal  $a_w$  at a lower level than that outside the cell, establishing a tendency for water to flow into the cell. This influx of water results in increasing hydrostatic pressure exerted by the cell membrane on the cell wall and is called turgor pressure or, simply, turgor. Turgor appears to be vital for the elongation of cells during division and, thus, maintenance of turgor is essential for bacterial growth (Koch, 1982). Osmotic stress decreases or abolishes turgor and, therefore, inhibits growth. DNA replication is also inhibited at low  $a_w$  (Meury, 1988). Gould and Measures (1977) reported the lower  $a_w$  limit for growth of *E. coli* to be 0.95 in laboratory media adjusted with NaCl and this was confirmed by Salter *et al.* (2000).

Loss in turgor *per se* does not appear to result in a loss in viability of bacterial cells, even under extreme conditions (Gould, 1989b). This suggests that, in response to some stresses, the processes of bacterial growth inhibition and inactivation are distinct, involving separate mechanisms that are defined by the intensity of the stress imposed. Conditions of low  $a_w$  have been widely used as an effective means of inactivating *E. coli* in foods, despite that the mode of action of this lethal agent has not been fully elucidated. Although some reviews exist that are entirely devoted to a description of bacterial inactivation (Leistner and Rödel, 1976; Troller and Christian, 1978), such contributions tend to focus on the effect of combining low  $a_w$  with other growth-limiting parameters rather than providing an account of the mechanisms involved. It is, however, apparent that all cellular reactions require an aqueous environment (Adams and Moss, 2000) and consequently the survival of a bacterial cell must also be dependent on the presence of water molecules within the

cytoplasm. Cellular sites that are damaged by low  $a_w$  conditions include the cell wall and the cell membrane (Mossel and Van Netten, 1984), however, whether such injury can be lethal, or damage to some other cellular site actually kills the cell, is unclear. One aspect of the low  $a_w$  effect on bacteria that has been better defined is the specific physiological response initiated by *E. coli* in an attempt to counteract osmotic disturbances and maintain normal cell functioning, termed osmoregulation.

### 1.3.2 Osmoregulatory Response

Given that the passage of water across the bacterial cell membrane is essentially passive, bacteria generally control the influx and efflux of solutes from the cell in response to osmotic stress. The main means by which *E. coli* achieves this is to lower cytosolic  $a_w$  by accumulating various solutes through active processes. This effectively decreases the  $a_w$  of the cytoplasm and thereby reduces further water efflux and contributes to the maintenance of turgor (Bremer and Krämer, 2000).

Within seconds of an osmotic shock, the internal potassium ion concentration of *E. coli* increases (Epstein, 1986). This occurs due to plasmolysis but also because of the active accumulation of potassium ions from the environment, which occurs via the two transport systems Trk and Kdp. The Trk transport system is constitutively expressed and is the main transporter of potassium ions in *E. coli* under optimal  $a_w$  conditions. The uptake of potassium by this system is increased upon osmotic stress (Jung *et al.*, 1997). The Kdp transport system is not expressed under normal circumstances but scavenges potassium ions when the low-affinity Trk transport system is not sufficient for the needs of the cell. That is, when the external concentration of potassium ions is low, when Trk is impaired due to mutation or when the environmental  $a_w$  is low. Unlike the Trk transport system, Kdp shows a

very high affinity for potassium ions but a slower rate of transport (Altendorf *et al.*, 1992). To maintain electroneutrality, the accumulation of potassium ions is balanced by accumulation of the anion glutamate via *de novo* synthesis (Dinnbier *et al.*, 1988; McLaggan *et al.*, 1994; Kempf and Bremer, 1998).

The immediate accumulation of potassium ions in response to low external  $a_w$  creates a highly ionic cellular environment that is detrimental to protein function and DNA-protein interactions. This initial response is, therefore, unsuitable when microorganisms are exposed to low  $a_w$  conditions for a prolonged period. In these instances, osmoregulation further involves the amassing of large amounts of organic molecules that contribute to cytoplasmic  $a_w$  but do not interfere with cell function (Csonka and Epstein, 1996). Because they are compatible with cell physiology, these compounds are called compatible solutes (Brown, 1976) and their accumulation forms the basis of the secondary response of *E. coli* to reduced  $a_w$ .

A variety of compatible solutes are accumulated by *E. coli* including carnitine, ectoine, the carbohydrate trehalose (via *de novo* synthesis) and the amino acids proline and glutamine (Galinski and Truper, 1994). Another potent osmoprotectant in *E. coli* is glycine betaine, more commonly referred to, simply, as betaine, which is a trimethylammonium compound widely available to bacteria, including those within foods (Landfald and Strøm, 1986; Larsen *et al.*, 1987; Gutierrez *et al.*, 1995). *E. coli* amasses this compatible solute predominantly through the uptake of exogenous betaine via the ProP and ProU transport systems. These systems are responsible for the accumulation of the majority of compatible solutes in *E. coli* and their expression is increased in response to low  $a_w$  conditions (Kempf and Bremer, 1998). When betaine is not exogenously available some strains of *E. coli* synthesise this compound from the precursors choline and betaine aldehyde (Strøm *et al.*, 1986).



This process is energy-demanding and does not occur when betaine is present in the environment due to down-regulation of the expression of one of the enzymes (BetB) involved in the synthesis pathway (Eshoo, 1988).

Further to the accumulation of solutes in the cytoplasm, changes in cell membrane lipid composition also occur in response to low  $a_w$  and such changes have been suggested to be part of the osmoregulatory sensing mechanism in *E. coli* (Russell and Kogut, 1985). Specifically, the levels of anionic lipids (e.g. diphosphatidylglycerol and phosphatidylglycerol) increase relative to their neutral, zwitterionic counterparts (e.g. phosphatidylethanolamine), which maintains the structure of the lipid bilayer and allows for proper functioning of the membrane in the low  $a_w$  environment (McGarrrity and Armstrong, 1975; Beales, 2004).

### **1.3.3 Meat and Meat Products**

Foodborne bacteria frequently encounter low  $a_w$  conditions in meat. The  $a_w$  of muscle is approximately 0.99 (Leistner and Rödel, 1976) but this is reduced due to the evaporation of water from the warm carcass surface and, later, during the chilling process to levels that may inhibit the growth of EHEC (Nottingham, 1982; Salter, 1998). The  $a_w$  of meat products is reduced from that of the fresh meat due to processing activities that cause water loss (via drying), or involve the addition of salts (usually NaCl) or sugars. The final  $a_w$  of meat products such as bologna sausage are typically around 0.97 and blood sausages tend to have a similar  $a_w$  value (Leistner and Rödel, 1976). Meat products that are not heated during processing and are consumed raw tend to have a much lower  $a_w$  because the preservation of such foods more heavily relies on the antimicrobial effect of  $a_w$ . As such, the final  $a_w$  of fermented sausages ranges from approximately 0.65 to 0.96,

with a modal value of about 0.91 (Leistner and Rödel, 1976). Despite not being heat treated, uncooked, comminuted fermented meat (UCFM) products have held a good safety record due primarily to the growth inhibition and/or inactivation effect of low  $a_w$  in combination with that caused by the low pH of the product.

In conclusion, it is well established that *E. coli* responds to a lowering of environmental  $a_w$  by osmoregulatory processes that accumulate solutes to avoid excessive water loss from the cell. The low  $a_w$ -induced inactivation of *E. coli* would, therefore, appear to occur when the osmoregulatory capacity of the cell is exceeded (Gould, 1989b), however, the exact mechanism(s) of this inactivation still remains to be defined. While the  $a_w$  of many foods are already manipulated as an effective means of food preservation, a better understanding of the mechanisms of action of low  $a_w$  and the responses of pathogenic bacteria to  $a_w$  levels relevant to food manufacture offers the potential to improve current food preservation techniques.

## Objectives

Outbreaks of illness caused by *E. coli* and associated with meat products with inherently low water activity values have demonstrated the ability of pathogenic *E. coli* to survive processes involved in meat manufacture. The aim of the work reported in this thesis is to improve the present understanding pertaining to the inactivation of *E. coli* in low water activity environments by:

1. **Characterising the kinetics of inactivation of *E. coli* in response to low water activity conditions relevant to foods such as uncooked, comminuted fermented meat.**

The inactivation of populations of *E. coli* exposed to low water activity (0.90) in laboratory media is analysed as well as that in response to low water activity and low pH, since the combination of such conditions can be of relevance to uncooked, comminuted fermented meat products. Some aspects of cellular injury that occur in *E. coli* exposed to these inimical conditions are described.

2. **Evaluating the rate of inactivation of *E. coli* in a laboratory-based system designed to mimic that during the processing of uncooked, comminuted fermented meat.**

A laboratory-based broth model is developed and used to systematically generate data pertaining to the inactivation of *E. coli* in uncooked, comminuted fermented meat. The results are analysed to reveal and quantify basic patterns of response. The findings are incorporated into a mathematical model that predicts the level of *E. coli* inactivation in uncooked, comminuted fermented meat products for use within the food industry.

**3. Determining the involvement of a self-mediated mechanism of bacterial cell death in response to low water activity environments.**

To provide insights into the mechanisms that mediate low water activity-induced inactivation of *E. coli*, the involvement of a specific system (*mazEF*), known to mediate the death of *E. coli* in response to some other inimical conditions, is assessed.

**4. Evaluating specific responses to lethal water activity stress in *E. coli*.**

A series of investigations are conducted to determine if *E. coli* induce a specific response when exposed to lethal water activity stress to enhance their ability to survive in those conditions. The survival characteristics of *E. coli* exposed to low water activity in the presence and absence of the compatible solute betaine is assessed to determine if the accumulation of this osmoprotectant prevents low  $a_w$ -induced inactivation. Further, protein expression in the surviving proportion of osmotically stressed populations of *E. coli* is examined by proteomic methods to ascertain the involvement of any stress-related proteins.

## **Chapter 2**

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# **Assessing Viability and Injury – Preliminary Investigations**

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## 2.1 INTRODUCTION

A major strategy for assuring the microbiological safety of foods relies upon various methods, including exposure to low  $a_w$ , that kill potential pathogens or inhibit their growth. Understanding the response of bacteria to such inimical processes is fundamental to food preservation and microbiological safety and has been studied extensively in relation to treatments of high temperature, low pH, low  $a_w$  and exposure to various toxins (reviewed by Storz and Hengge-Aronis, 2000). In particular, assessment of the actual number of microorganisms able to survive a specific stress (i.e. those that remain viable) is central to determining whether that treatment can produce a food product that is safe for consumption.

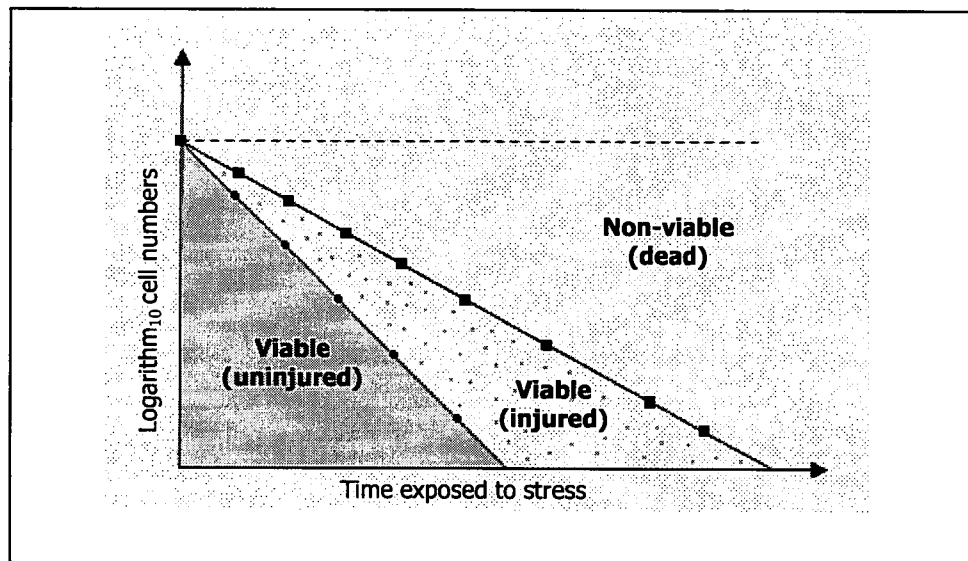
A bacterial cell is described as 'viable' if it can grow to detectable levels when provided with a favourable environment. When a microorganism is unable to do so, it is considered non-viable or dead. The term 'viability' is used to describe the proportion of the bacterial population that is viable. The most common method employed for determining bacterial viability assesses the capacity of cells to replicate and thereby form colonies on agar plates (Postgate, 1969). More recently, cytological assays that use criteria other than replication to differentiate between viable and non-viable cells have been developed (Barer and Harwood, 1999). These techniques, such as flow cytometric analysis of stained cellular components or assessment of enzyme activity, offer the potential to be far more rapid because they do not require lengthy incubation periods typical of culture-based methodologies and, in some cases, may be less labour intensive. Unfortunately, the validity of such methods as a means of accurately determining microbial inactivation has yet to be satisfactorily shown (Kell *et al.*, 1998; Barer and Harwood, 1999) and, at this time, culture-based enumeration remains the most

reliable method to determine viability (Kell *et al.*, 1998). Thus, in a functional sense, culturability is considered synonymous with viability (Postgate, 1969).

Microorganisms that survive an inimical process may be injured, either metabolically, structurally or both, and as a result are more fastidious in their requirements for growth (Postgate, 1969). Evidence suggests that these injured cells retain their capacity to cause disease (Sorrells *et al.*, 1970), particularly when provided with an environment in which the damage can be repaired (Collins-Thompson *et al.*, 1973; Busta, 1976), and, thus, it is important that methods used to assess bacterial viability allow both uninjured and the more fastidious injured cells to be recognised. An enumeration protocol that fails to recover some or all of the injured population will underestimate the number of viable microorganisms able to survive a given stress. Thus, the lethality of that treatment will be overstated leading to inaccurate and potentially dangerous conclusions.

In practice, metabolically injured cells exhibit an inability to grow on nutrient-poor media that would normally support their growth but continue to form colonies on nutrient-rich, non-selective agar. Conversely, cells that have sustained structural injury do not grow to detectable levels on selective media but will grow on non-selective agars (Beuchat, 1978). The level of bacterial injury in response to an inimical process can, therefore, be measured in an indirect manner by culture-based methodologies. This requires that both the total viable population (injured and uninjured cells) and the uninjured population can be quantified, which is achieved by plating dilutions on two media, as shown in Figure 2.1. An example of the usefulness of such an approach is in the quantification of injury in bacterial cells using media containing bile salts as a selective agar. When a stress damages cellular enzymes that normally break down toxins, disrupts efflux systems that

ordinarily export bile salts from the cell, and/or compromises the outer membrane allowing high concentrations of bile salts to penetrate the cell, bacterial growth is inhibited by bile salts (Beuchat, 1978; Gunn, 2000). Thus, agar containing bile salts select against those injured cells and allow the level of injury in bacterial populations to be measured.



**Figure 2.1** The viability and proportion of injured and uninjured cells within the viable subpopulation of a hypothetical bacterial culture exposed to a particular stress. All viable cells are recovered on the non-selective agar (■), while only uninjured cells are recovered on the selective agar (●); the difference in numbers recovered on these two media represents the structurally injured population. Adapted from Mackey (2000).

To present an accurate account of the loss of viability and the level of injury in populations of *E. coli* exposed to osmotic and/or acid stress in following chapters, the aim of the current chapter was to determine factors that influence bacterial inactivation. From this, a suitable methodology was developed. Variables analysed included the preparation of the experimental inoculum, the environment in which the bacterial population was exposed to non-thermal stress and the effect of specific



environments during culture-based enumeration to measure the proportion of injured cells.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Bacterial Strains, Reagents, Media, Solutions and Equipment**

Details of bacterial strains, chemical reagents, bacteriological media, solutions and equipment (including software) are given in Appendix A. All experiments described in this chapter (as well as those in Chapters 3, 4 and 6) employed the non-pathogenic *E. coli* M23 OR:H-. This strain has a temperature growth range of 7.8 – 48°C and a  $a_w$  growth range of 0.95 – 0.999 (Salter *et al.*, 1998). Under growth-permitting conditions, Salter *et al.* (1998) found no significance difference in the growth rate and response to NaCl and temperature between *E. coli* M23 and pathogenic strains such as *E. coli* O157:H7 and O157:H-. Brown *et al.* (1997) found that *E. coli* M23 responds to acid stress similarly to some of the most virulent strains of pathogenic *E. coli* (e.g. O157:H-). In that study, the inactivation profile of *E. coli* M23 in response to pH 3.0 indicated that this non-pathogenic strain is inactivated similarly to *E. coli* O157:H- and is more acid resistant than another pathogenic strain (*E. coli* O81:H-, R91). Therefore, the use of *E. coli* M23 OR:H- was considered suitable for this study.

### **2.2.2 General Methods**

#### **2.2.2.1 Preparation of Stationary Phase Populations of *Escherichia coli***

Five colonies of *E. coli* M23 on tryptone soya agar (TSA; A.1.1) were inoculated to 80 mL tryptone soya broth (TSB) in a 250 mL Erlenmeyer flask and incubated

statically at 37°C for 24 ( $\pm$  0.25) hours, to achieve a viable count of approximately 9.0 log CFU.mL<sup>-1</sup>.

#### **2.2.2.2 Preparation of Low Water Activity and/or Low pH Broth**

A measured amount (typically 6-8 g) of NaCl (sterilised with UV) was added to 50 mL of specified medium in a 250 mL Erlenmeyer flask to yield a final  $a_w$  0.900 ( $\pm$  0.003). The pH of a separate 50 mL volume of specified medium in a 250 mL Erlenmeyer flask was aseptically adjusted to 3.50 ( $\pm$  0.05) using 10 M HCl. Where both low  $a_w$  and low pH were required, the  $a_w$  was adjusted prior to the pH. Flasks were incubated at 25°C in a water bath for several hours prior to inoculation to equilibrate to temperature.

#### **2.2.2.3 Harvesting Escherichia coli and Inoculation to Broth**

Aliquots (5 mL) of stationary phase populations (2.2.2.1) of *E. coli* M23 were transferred to 15 mL sterile tubes. Cells were pelleted by centrifugation at 5000 rpm for 10 minutes at room temperature (RT) in a Universal 16A centrifuge. The supernatant was removed using a pipette and the pellet washed in 5 mL minimal minerals medium containing 0.1% D-glucose (MM) and centrifuged as above. The supernatant was removed by pipetting and the cells transferred to 50 mL low  $a_w$  and/or low pH broth prepared as in Section 2.2.2.2. Specifically, a 1 mL aliquot of the low  $a_w$  and/or low pH broth was removed from the flask and used to resuspend the cell pellet. This cell suspension was then returned to the flask, thus providing a 1/10 dilution of the original stationary phase population. Flasks were incubated at 25°C in a water bath with shaking at 60 oscillations per minute. Immediately following inoculation and at the end of the treatment, a 2 mL aliquot was withdrawn for  $a_w$  and pH measurements.

#### **2.2.2.4 Enumeration of Viable Cells and Construction of Survival Curves**

The viability of each population was estimated by culture-based enumeration immediately prior to the inimical treatment and either at regular intervals throughout or at a specific time point as described. Specifically, 100  $\mu\text{L}$  aliquots were removed and serially diluted in diluent. Samples (50 or 250  $\mu\text{L}$  volumes) were surface plated using a spiral plater onto brain heart infusion agar supplemented with 0.1% sodium pyruvate (BHA-P). Plates were incubated at 37°C for 14 ( $\pm$  0.5) hours and CFU were quantified using an image scanner and CIA-BEN software. Survival curves were constructed by plotting the  $\log_{10}$  of the CFU.mL<sup>-1</sup> against time. For convenience, when viable cells could not be detected in a 50  $\mu\text{L}$  volume of an undiluted sample, the number of viable cells was plotted as 1.30 log CFU.mL<sup>-1</sup>. Where a 250  $\mu\text{L}$  volume was plated, an undetectable number of viable cells was plotted as 0 log CFU.mL<sup>-1</sup>.

### **2.2.3 Assessment of Laboratory Systems Used to Describe Non-Thermal Inactivation Kinetics**

#### **2.2.3.1 Inoculum Preparation**

*E. coli* M23 colonies grown on TSA by incubation at 37°C for 14 hours (A.1.1) were used to prepare stationary phase populations (2.2.2.1) either immediately or following storage at 4°C for 48 hours. The populations were inoculated to MM supplemented with 2 mM betaine (MM-B) and were exposed to low  $a_w$  [ $a_w$  0.90, 25°C, MM-B] or low pH [pH 3.5, 25°C, MM-B] (2.2.2.3) for up to 813.75 and 652.58 hours respectively. The number of viable cells was estimated periodically by culture-based enumeration (2.2.2.4).

### **2.2.3.2 Suspension Medium for Stress Treatments**

Stationary phase populations (2.2.2.1) of *E. coli* M23 were exposed to low  $a_w$  [ $a_w$  0.90, 25°C], low pH [pH 3.5, 25°C], or low  $a_w$  and pH [ $a_w$  0.90, pH 3.5, 25°C] (2.2.2.3) in four medium types: MM-B, TSB, nutrient broth (NB), and Luria-Bertani broth (LB). Immediately prior to the treatment, and at regular intervals throughout, the number of viable cells was determined by culture-based methods (2.2.2.4).

### **2.2.4 Evaluation of the Diluent Used for the Enumeration of Uninjured *Escherichia coli* Exposed to Non-Thermal Stress**

Stationary phase populations (2.2.2.1) of *E. coli* M23 were exposed to low  $a_w$  [ $a_w$  0.90, 25°C] or low pH [pH 3.5, 25°C] in TSB (2.2.2.3). At three separate time points (i.e. after approximately 30 and 215 hours of treatment respectively) aliquots were removed and serially diluted in diluent. Volumes (50  $\mu$ L) of the  $10^{-2}$ ,  $10^{-1}$  and undiluted ('neat') samples were surface plated in triplicate to brain heart infusion agar supplemented with 0.15% bile salts No. 3 (BHA-BS) using a spiral plater. When required, the remainder of the cell suspension was pelleted by centrifugation at 10 000 rpm for 10 minutes at 4°C in a Microcentrifuge 5417R and the pellet resuspended in an equal volume of diluent, MM, MM with  $a_w$  0.90 (2.2.2.2), TSB or TSB with  $a_w$  0.90 (2.2.2.2). Samples were similarly plated onto BHA-BS. The plates were incubated at 37°C for 14 ( $\pm$  0.5) hours and the number of CFU determined using an image scanner and CIA-BEN software. The log number of CFU.mL<sup>-1</sup> (i.e. viable cells without injury) for each dilution was determined. The data were analysed for significant differences ( $P \leq 0.01$ ) via multiple Student's *t*-tests (two-sample assuming equal variances) using Microsoft® Excel.

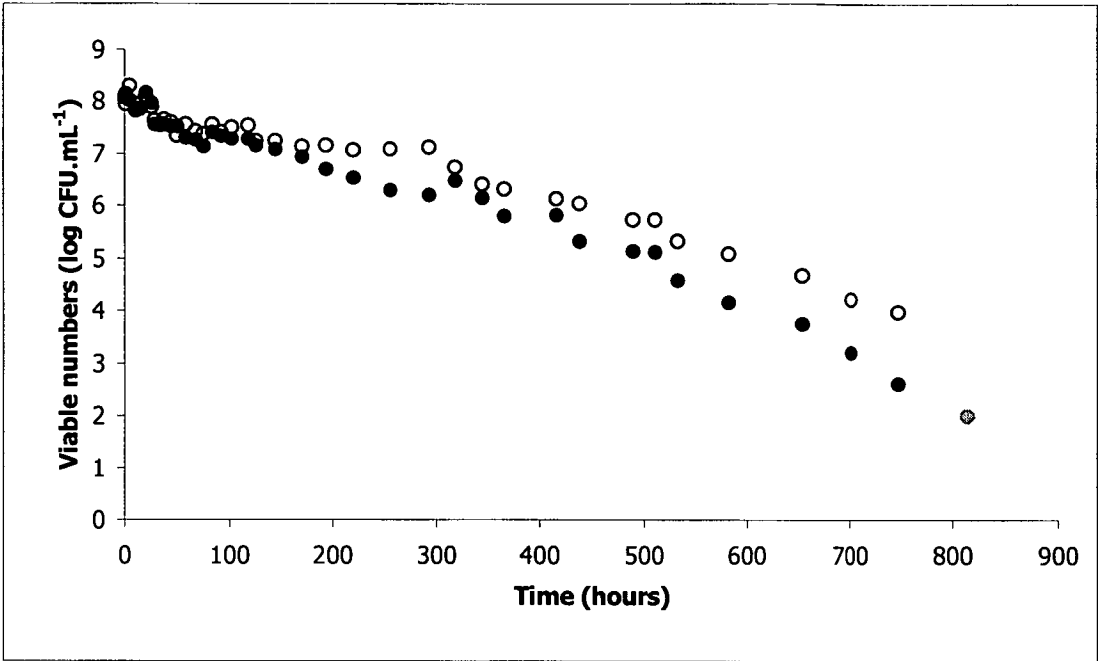
For the construction of survivor curves, stationary phase populations (2.2.2.1) of *E. coli* M23 were exposed to low  $a_w$  [ $a_w$  0.90, 25°C] in MM-B or TSB (2.2.2.3). Immediately prior to the inimical treatment, and at regular intervals throughout, 100  $\mu$ L volumes were withdrawn from the population and serially diluted in diluent and 50 or 250  $\mu$ L volumes were surface plated to BHA-BS as described above. The remainder of the cell suspension was pelleted as described above and the pellet resuspended in an equal volume of diluent. Samples were similarly surface plated onto BHA-BS. The plates were incubated as described above and the log number of CFU.mL<sup>-1</sup> was plotted against time. The minimum level of detection was similar to that described in Section 2.2.2.4.

## **2.3 RESULTS**

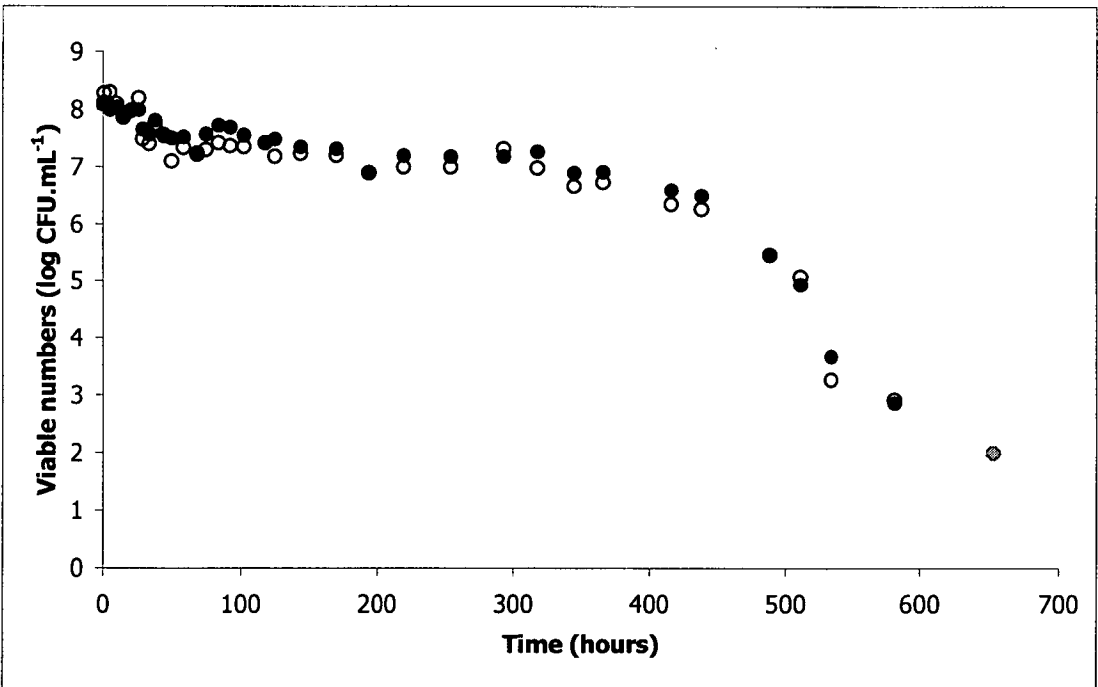
### **2.3.1 Assessment of Laboratory Systems Used to Describe Non-Thermal Inactivation Kinetics**

#### **2.3.1.1 Inoculum Preparation**

The method employed for the preparation of stationary phase populations relied upon the transfer of five colonies of *E. coli* M23 from TSA to TSB. Colonies were either transferred immediately (unstressed) or the plate stored at 4°C for 48 hours (cold-stressed) before transfer to TSB. The inactivation of each population in response to low  $a_w$  [ $a_w$  0.90, 25°C, MM-B] and low pH [pH 3.5, 25°C, MM-B] is shown in Figure 2.2 and 2.3 respectively. The data are representative of two separate experiments.



**Figure 2.2** Inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 (NaCl as humectant) at 25°C in MM-B using unstressed (●) or cold-stressed (○) colonies for preparation of the experimental inoculum. Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

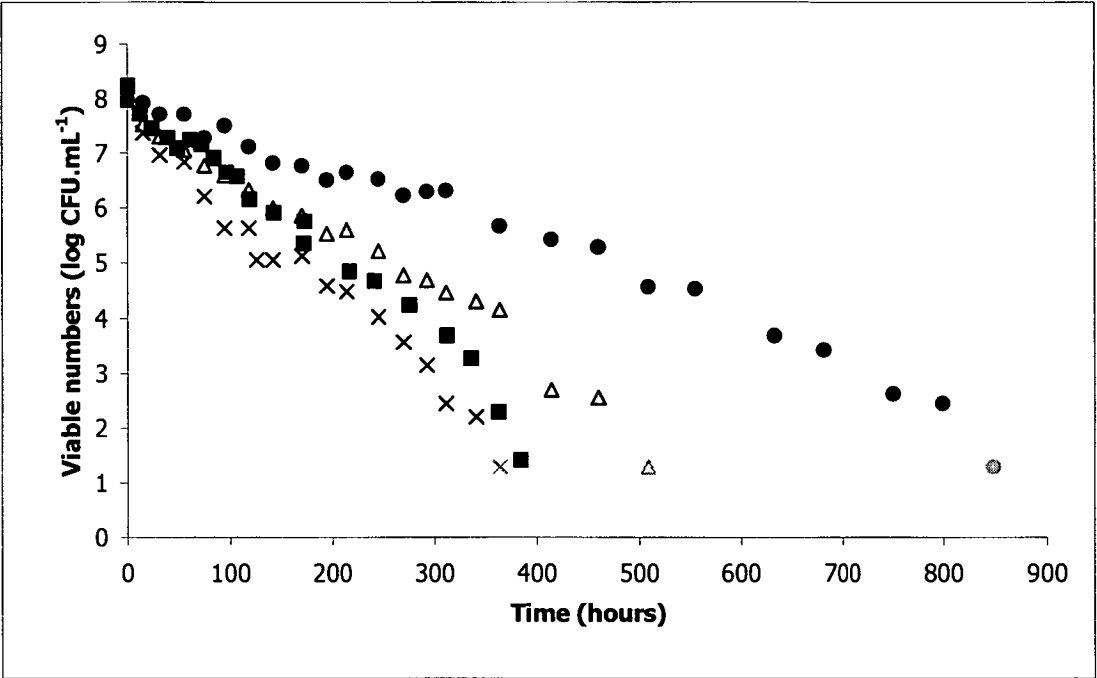


**Figure 2.3** Inactivation kinetics of *E. coli* M23 in response to pH 3.50 (HCl as acidulant) at 25°C in MM-B using unstressed (●) or cold-stressed (○) colonies for preparation of the experimental inoculum. Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

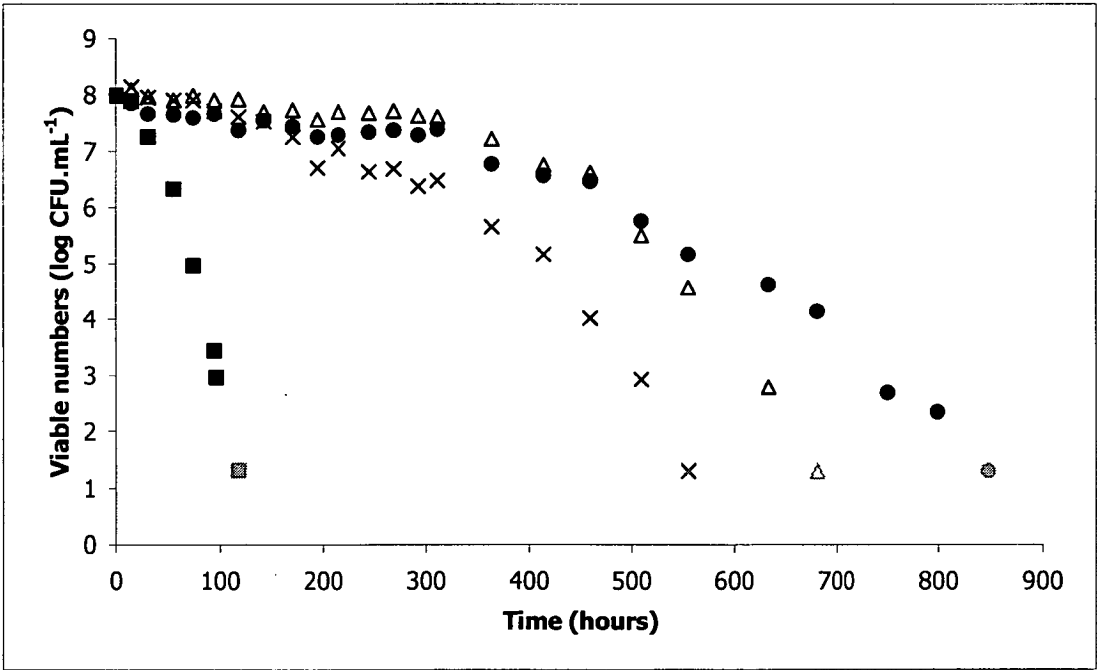
Whether cultured from unstressed or cold-stressed colonies, the populations of *E. coli* M23 exposed to low  $a_w$  conditions showed almost identical inactivation up until 170.58 hours of treatment (i.e. the difference did not exceed  $0.29 \log \text{CFU.mL}^{-1}$  at any sampling time). After this point, however, the unstressed population showed a lower level of survival than the cold-stressed population until 813.75 hours when the minimum level of detection ( $1.30 \log \text{CFU.mL}^{-1}$ ) was evident in both populations. Between 172 and 813 hours, the number of viable cells in the population cultured from cold-stressed colonies was  $> 0.29 \log \text{CFU.mL}^{-1}$  than that in the unstressed population for 14 of the 16 sampling times (i.e. 87.5% of cases), indicating a consistent trend. Conversely, the viability of populations of *E. coli* M23 prepared from either unstressed or cold-stressed colonies and exposed to low pH were very similar over the entire course of the experiment (i.e. the number of viable cells for each population varied by no more than  $0.42 \log \text{CFU.mL}^{-1}$  at any sampling time). Collectively, these results indicate that the use of cold-stressed colonies in the preparation of stationary phase populations of *E. coli* alter the observed inactivation kinetics upon exposure to low  $a_w$  but not low pH.

### **2.3.1.2 Suspension Medium for Stress Treatments**

Previous studies within this laboratory have most commonly used TSB to investigate the response of bacteria to non-thermal (acid and osmotic) stress (Shadbolt *et al.*, 2001; Brown, 2002; Shadbolt, 2004). In instances where a component present in TSB needs to be excluded, MM is usually used. To determine the influence of different medium types on *E. coli* inactivation a comparison of the inactivation kinetics of diluted stationary phase populations of *E. coli* M23 in response to low  $a_w$  [ $a_w$  0.90, 25°C], low pH [pH 3.5, 25°C] or low  $a_w$  and low pH [ $a_w$  0.90, pH 3.5, 25°C] in MM-B, TSB, NB and LB was made during two temporally distinct experiments. The results of single experiments are depicted in Figures 2.4 - 2.6.

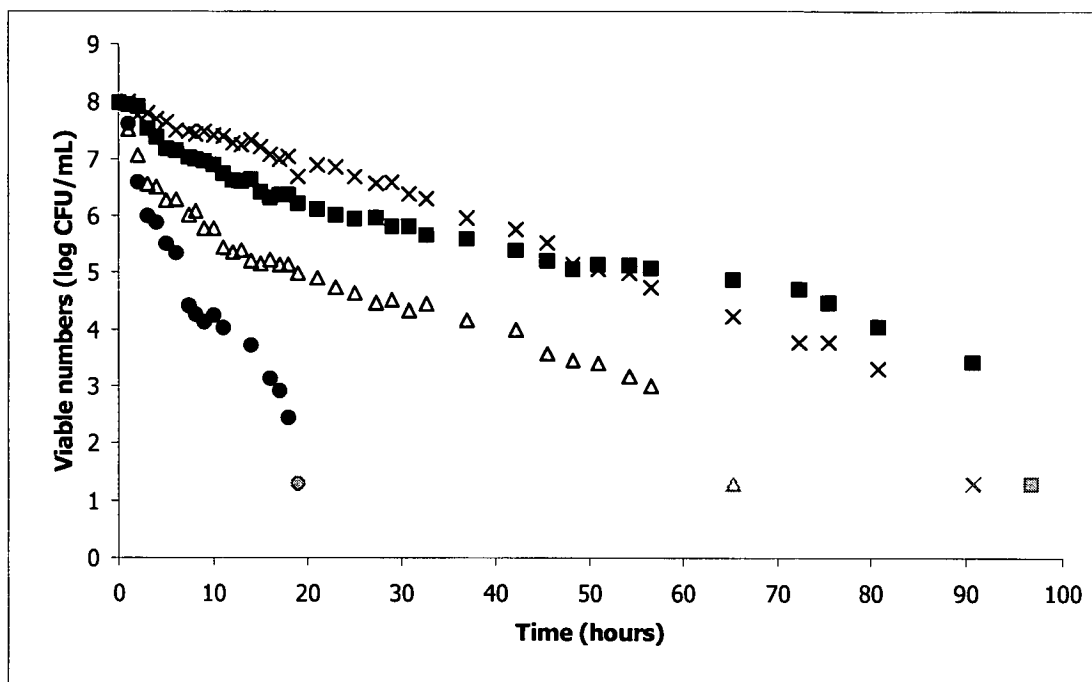


**Figure 2.4** Inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 (NaCl as humectant) at 25°C in MM-B (●), TSB (■), NB (×) or LB (△). Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure 2.5** Inactivation kinetics of *E. coli* M23 in response to pH 3.5 (HCl as acidulant) at 25°C in MM-B (●), TSB (■), NB (×) or LB (△). Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.





**Figure 2.6** Inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 and pH 3.5 (NaCl as humectant and HCl as acidulant) at 25°C in MM-B (●), TSB (■), NB (×) or LB (△). Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

In response to low  $a_w$ , *E. coli* M23 showed the best survival in MM-B, and was detected ( $> 1.30 \log \text{CFU.mL}^{-1}$ ) for 799 hours of treatment (see Figure 2.4). This survival was markedly greater than in the other three medium types. For example, when viable cells could not be detected in any of the populations exposed to low  $a_w$  in TSB, NB or LB, a large number of viable cells ( $4.56 \log \text{CFU.mL}^{-1}$ ) remained for the population in MM-B. The poorest survival was evident in NB where viable numbers fell below the detectable limit within 364 hours. Populations of *E. coli* M23 exposed to  $a_w$  0.90 in TSB and LB showed similar inactivation kinetics for approximately 75 hours (i.e. viability differed by less than  $0.50 \log \text{CFU.mL}^{-1}$  at any time point), after which time the population in TSB displayed a greater rate of inactivation and viable numbers were detected for 383 hours, compared to 460 hours for the population in LB.

Populations of *E. coli* M23 exposed to pH 3.5 in different media exhibited distinct patterns of inactivation. As shown in Figure 2.5, populations in TSB showed a greater rate of inactivation compared to the other medium types tested, and the number of viable cells fell below the detectable level ( $1.30 \log \text{CFU.mL}^{-1}$ ) after 97 hours. At this time, the number of viable cells in *E. coli* M23 populations exposed to low pH in MM-B, NB and LB had not decreased from their initial numbers by more than  $1.0 \log \text{CFU.mL}^{-1}$ . For approximately 400 hours, the population suspended in LB with low pH showed the highest level of survival but after this time, the inactivation rate increased and viable cells could not be detected at 681 hours. When suspended in NB with low pH, viable *E. coli* M23 cells were detected for 509 hours whereas the population suspended in MM-B survived for the longest period of time (i.e. viable cells were detected for 799 hours).

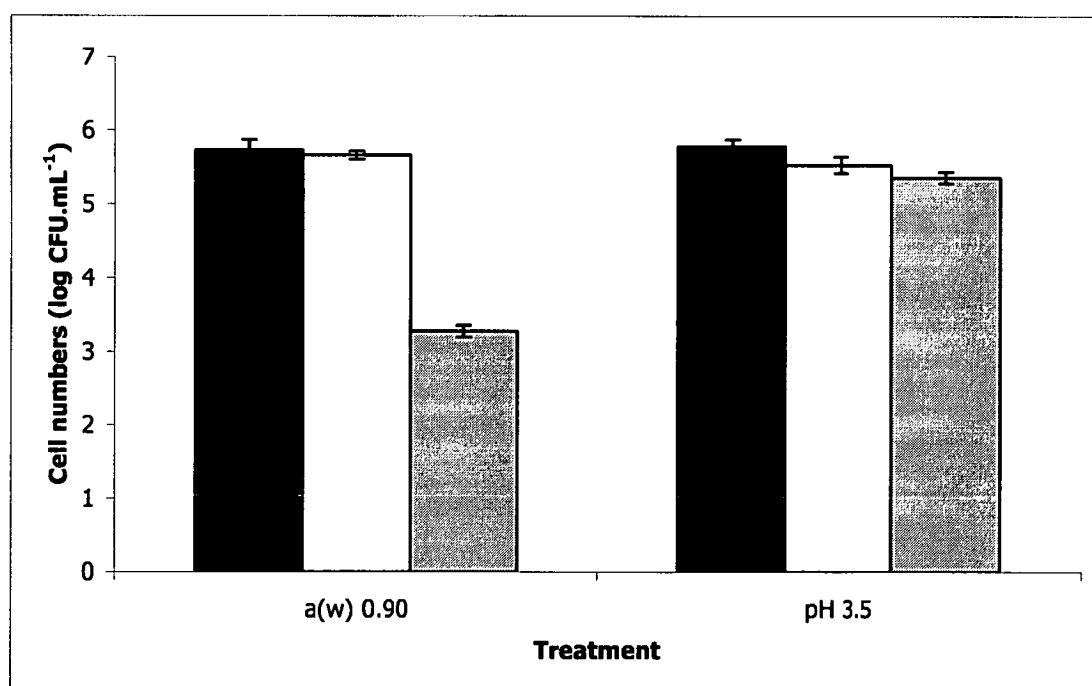
Upon exposure to both low  $a_w$  (0.90) and low pH (3.5), *E. coli* M23 populations were inactivated as depicted in Figure 2.6. The rate of inactivation was greatest in MM-B, where viable numbers were undetectable after 19 hours. Better survival was seen in LB, where the lowest detectable limit ( $1.30 \log \text{CFU.mL}^{-1}$ ) was not reached until 65 hours. The survival rate was greatest in NB and TSB cultures. Inactivation rate in NB was constant, whereas the inactivation of *E. coli* M23 in TSB was initially more rapid than that in NB. After 48 hours, however, the number of viable *E. coli* M23 was greater in the TSB than in NB and viable cells were detectable up until 91 and 81 hours respectively.

From Figures 2.4 – 2.6 it can be seen the combination of  $a_w$  0.90 and pH 3.5 in MM-B enhances inactivation of *E. coli* M23 compared to either of the individual stresses (i.e. the time taken to reduce the population to the minimum level of detection is less). In direct contrast, the combination of the two stresses in TSB did not

enhance microbial inactivation because the kinetics were very similar to the low pH-induced response. Exposure of populations to low  $a_w$  and pH in both NB and LB enhanced the inactivation of *E. coli* M23 in comparison to the individual stresses but this effect was not as pronounced as in MM-B.

### 2.3.2 Evaluation of the Diluent Used for the Enumeration of Uninjured *Escherichia coli* Exposed to Non-Thermal Stress

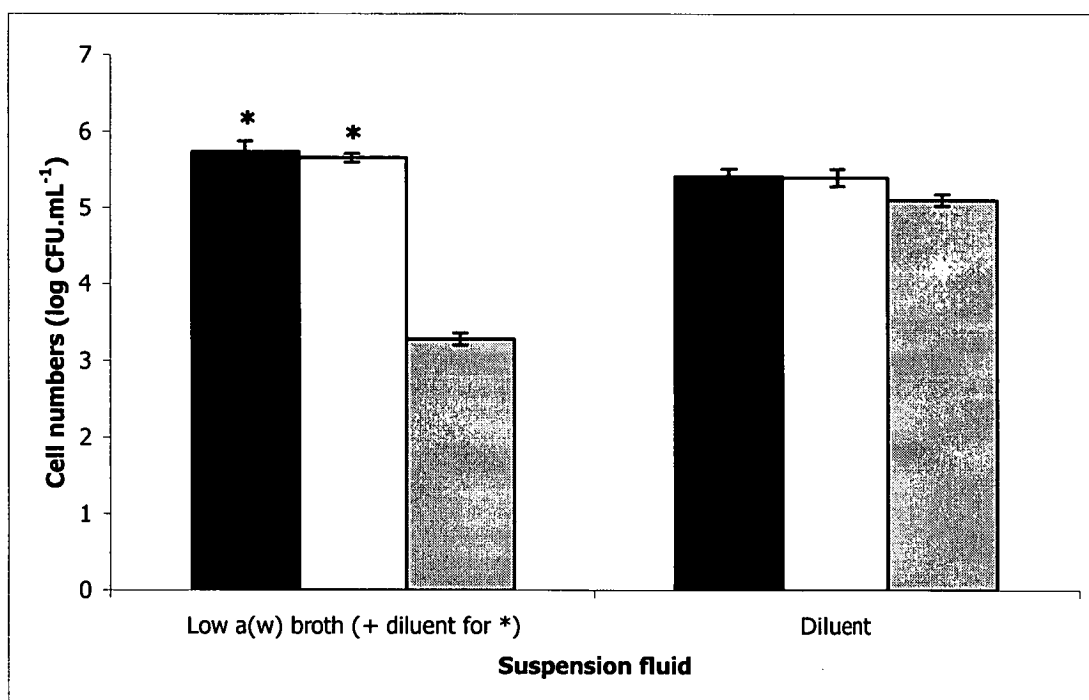
Enumeration of the uninjured proportion of populations of *E. coli* M23 exposed to low  $a_w$  [0.90, 25°C, TSB] or low pH [pH 3.50, 25°C, TSB] was determined by plating two diluted ( $10^{-1}$  and  $10^{-2}$ ) samples and one undiluted ('neat') sample of the culture subjected to the stress on BHA-BS in triplicate. The average number of cells recovered on BHA-BS at a single time point is shown in Figure 2.7.



**Figure 2.7** The number of *E. coli* M23 recovered on BHA-BS using three dilutions (■  $10^{-2}$ ; □  $10^{-1}$ ; or ▨ undiluted). Populations of *E. coli* M23 were exposed to  $a_w$  0.90 (NaCl as humectant) or pH 3.5 (HCl as acidulant) at 25°C in TSB for 31.25 and 216.75 hours respectively. Cells were recovered and enumerated on BHA-BS in triplicate at 37°C. Standard deviation (n=3) is shown.

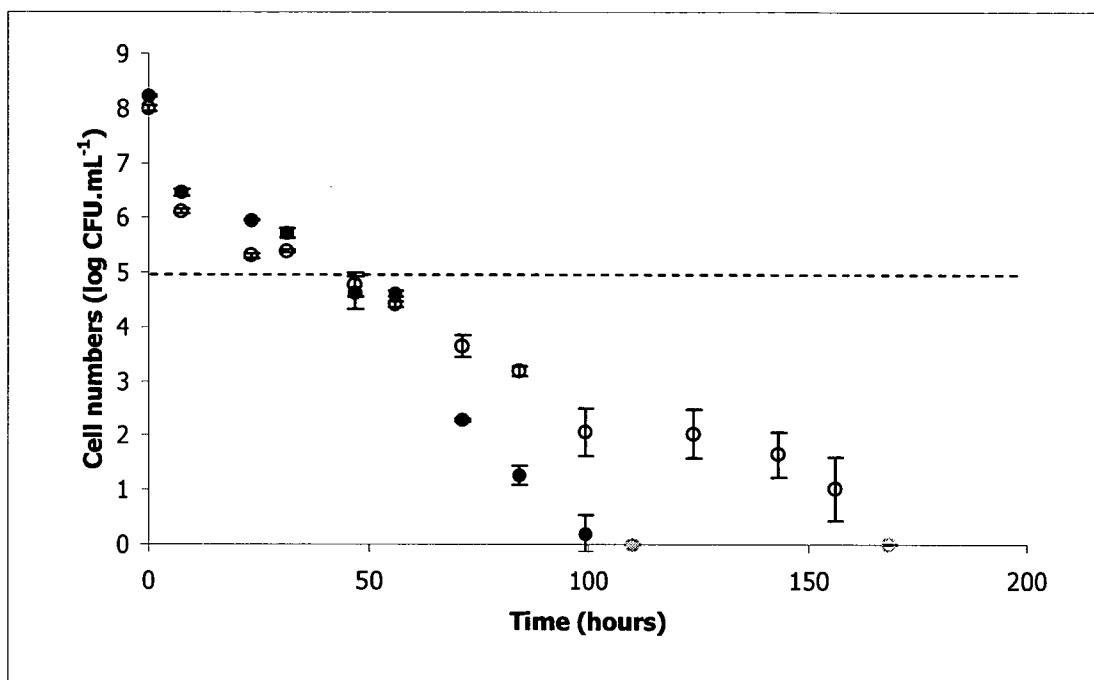
The significance of the data given in Figure 2.7 was tested by multiple Student's *t*-tests, which indicated that the recovery of low  $a_w$ -treated cells was significantly reduced on the neat plate in comparison to the two diluted samples ( $P \leq 0.01$ ). The same pattern was observed at another two time points. In contrast, the recovery of low pH-treated cells on BHA-BS using neat or diluted samples differed significantly in only one instance (i.e. between the neat and the  $10^{-2}$  dilution removed at a single time point as depicted in Figure 2.7) during evaluation at three different times. Thus, it appears that the  $a_w$  of the suspension fluid can significantly influence the recovery of cells on BHA-BS, but the pH does not necessarily do so.

The effect of the low  $a_w$  TSB on cell recovery was assessed by centrifuging the cell suspension and resuspending the pellet in an equal volume of diluent and enumerating *E. coli* M23 cells by plating on BHA-BS. The results from a single sample (with those for the low  $a_w$  broth previously presented in Figure 2.7) are depicted in Figure 2.8 and are representative of three sampling times. These results show that removal of the low  $a_w$  TSB from the neat sample by the method described increased the recovery of uninjured *E. coli* cells on BHA-BS. The results further illustrate that the method employed for removing the low  $a_w$  TSB in itself caused a reduction in the number of viable cells recovered (as seen by comparing that data for either the  $10^{-2}$  or  $10^{-1}$  samples). Multiple Student's *t*-tests determined this to be of statistical significance ( $P \leq 0.01$ ). This is likely to be due to loss of cells during removal of the supernatant or perhaps cells were killed or sustained injury due to the additional time that they were exposed to low  $a_w$  before the broth was removed.



**Figure 2.8** The number of *E. coli* M23 recovered on BHA-BS in the presence and absence of low  $a_w$  TSB using 3 dilutions (■  $10^{-2}$ ; □  $10^{-1}$ ; or ▨ undiluted). Populations of *E. coli* M23 were exposed to  $a_w$  0.90 (NaCl as humectant) at 25°C in TSB for 31.25 hours. Cells were recovered and enumerated on BHA-BS in triplicate at 37°C. Standard deviation (n=3) is shown.

To determine whether the modification of the sampling handling procedure (i.e. to remove the low  $a_w$  broth from neat samples prior to plating on BHA-BS) would provide a better description of the level of injury within osmotically stressed populations of *E. coli*, both methods were compared at regular intervals over an increased period of time. A population of *E. coli* M23 was exposed to low  $a_w$  [ $a_w$  0.90, 25°C, TSB] and the number of uninjured cells was determined as previously described. During sample handling, the low  $a_w$  TSB was either left in the suspension fluid or removed by centrifugation and resuspension of the pellet in normal diluent. Results are shown in Figure 2.9.

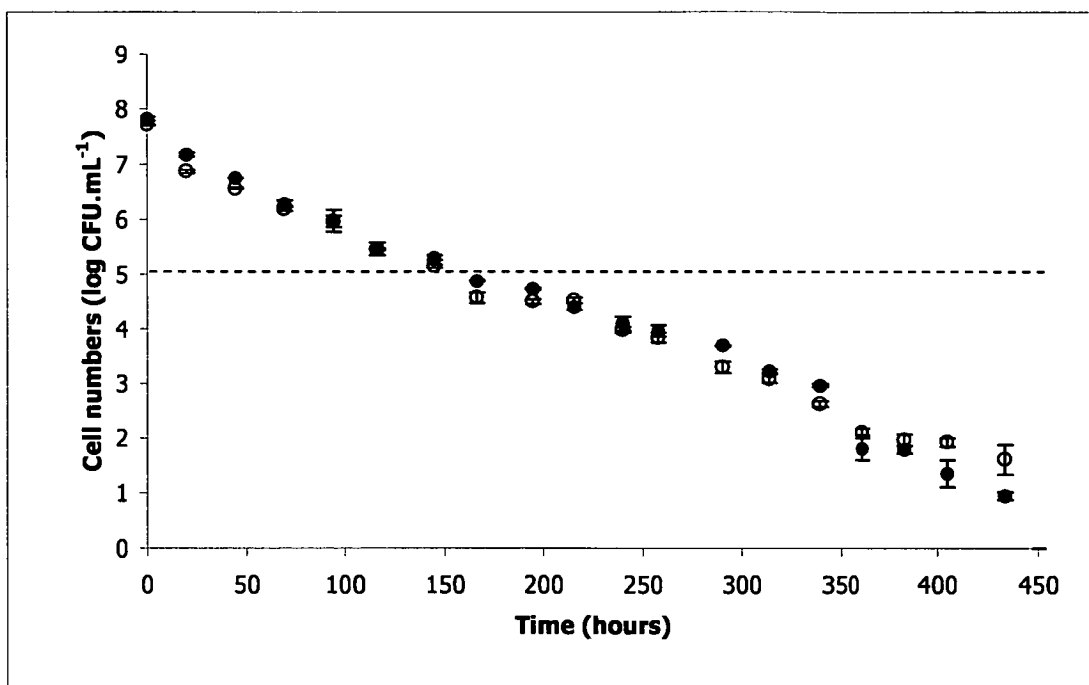


**Figure 2.9** The number of *E. coli* M23 recovered on BHA-BS following exposure to  $a_w$  0.90 (NaCl as humectant) at 25°C in TSB in the presence (●) and absence (○) of low  $a_w$  TSB. Grey data points indicate that the number of viable cells was below the minimum level of detection. Cells were recovered and enumerated on BHA-BS in triplicate at 37°C. Standard deviation ( $n=3$ ) is shown. All data below the dotted line used an undiluted sample to determine the number of cells (see text for details).

Initially, the removal of the low  $a_w$  TSB from the suspension fluid of diluted samples during sample handling did not substantially alter the number of cells recovered and enumerated on BHA-BS. However, as viable numbers decreased to a point where the neat sample was included in the measurement of viable numbers (i.e. at  $< 5.0$  log CFU.mL<sup>-1</sup>) the removal of the low  $a_w$  broth caused the number of cells recovered and enumerated to increase. When cell numbers were between 4.0 and 5.0 log CFU.mL<sup>-1</sup>, the number of cells was the average of that enumerated using three dilutions ( $10^{-2}$ ,  $10^{-1}$  and neat). Because the recovery of uninjured cells within the diluted samples was not substantially altered by the presence of low  $a_w$  TSB (as shown in Figure 2.7), the low recovery on the neat plate using this method did not

substantially alter the final number. However, as the number of viable cells decreased and could not be detected using the diluted samples (i.e. after 85 hours), the discrepancy in recovery of uninjured cells when the low  $a_w$  broth was either present or absent within the neat sample became important. In this case, the removal of the low  $a_w$  broth from the suspension fluid caused a dramatic increase in the number of cells recovered (at least 1 log CFU.mL<sup>-1</sup> at each sampling time). As a result, the subpopulation of uninjured *E. coli* M23 was shown to persist for up to 169 hours of low  $a_w$  exposure whereas the method that retained low  $a_w$  TSB in the suspension fluid indicated that uninjured *E. coli* M23 did not exist (at detectable levels) beyond 100 hours.

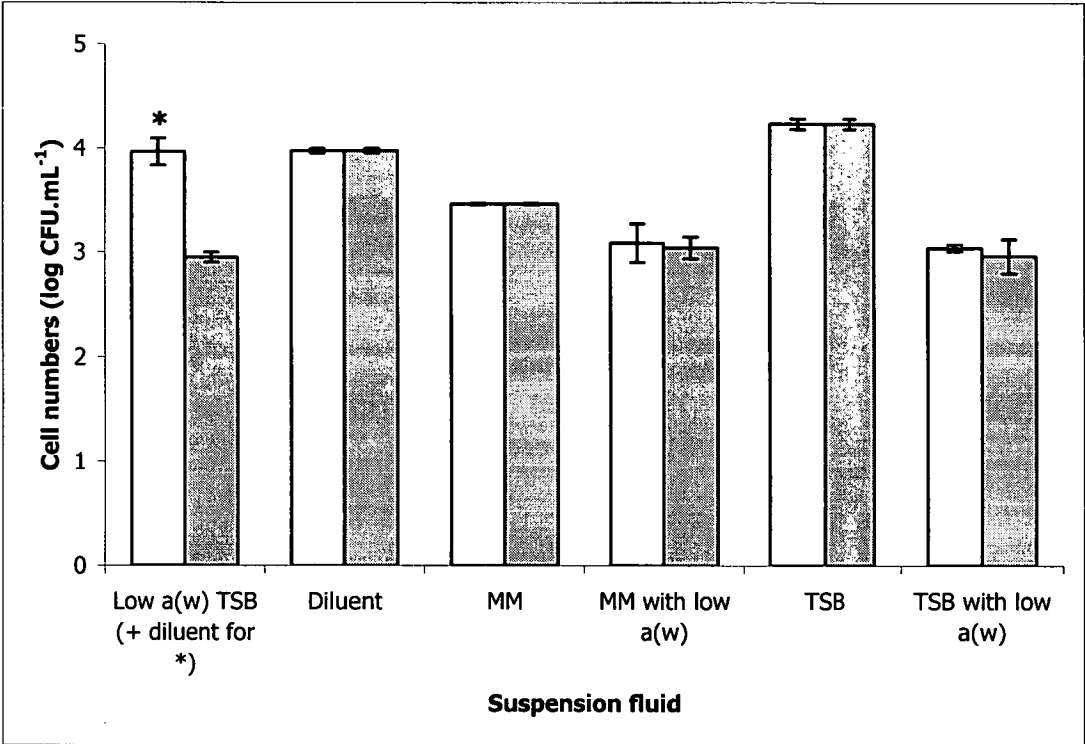
Some future experiments, described in subsequent chapters, were anticipated to require the exposure of *E. coli* populations to low  $a_w$  in MM-B rather than TSB. Consequently, the effect of the removal of the low  $a_w$  MM-B from the suspension fluid on enumeration of the uninjured population of *E. coli* M23 was assessed. Similar to the procedures described above, the uninjured proportion of a population of *E. coli* M23 exposed to low  $a_w$  in MM-B [ $a_w$  0.90, 25°C, MM-B] was determined by enumeration on BHA-BS using inocula that contained or did not contain low  $a_w$  MM-B. Figure 2.10 illustrates the findings and shows that the maximum difference in recovery between the two methods at a single time point was 0.66 log CFU.mL<sup>-1</sup>. This suggests that, in this case, the presence of low  $a_w$  MM-B does not influence the estimate of the number of uninjured *E. coli* M23.



**Figure 2.10** The number of *E. coli* M23 recovered on BHA-BS following exposure to  $a_w$  0.90 (NaCl as humectant) at 25°C in MM-B in the presence (●) and absence (○) of low  $a_w$  MM-B. Grey data points indicate that the number of viable cells was below the minimum level of detection. Cells were recovered and enumerated on BHA-BS at 37°C. Standard deviation ( $n=3$ ) is shown. All data below the dotted line used an undiluted sample to determine the number of viable cells.

Results presented above indicated that the recovery of uninjured *E. coli* M23 cells in neat samples was hindered by the presence of low  $a_w$  TSB but not low  $a_w$  MM-B. These findings prompted further investigations into the effect of suspension fluid composition on cell recovery. Samples removed from a population of *E. coli* M23 exposed to low  $a_w$  in TSB [ $a_w$  0.90, 25°C, TSB] for 34.58 hours were plated to BHA-BS. The suspension fluid used in each case was either unmodified (and, therefore, included the low  $a_w$  TSB in which the cells were suspended for the 34.58 hours of treatment) or the low  $a_w$  TSB was removed by centrifugation and the cells were resuspended in fresh solutions of diluent, MM, MM with low  $a_w$  (0.90), TSB or TSB with low  $a_w$  (0.90). Results of a single experiment are presented in Figure 2.11. The same pattern was observed during a separate experiment.





**Figure 2.11** The number of *E. coli* M23 recovered on BHA-BS in different suspension fluids using 2 dilutions (□ 10<sup>-1</sup>; or ■ undiluted). Populations of *E. coli* M23 were exposed to a<sub>w</sub> 0.90 (NaCl as humectant) at 25°C in TSB for 34.58 hours. Cells were recovered and enumerated on BHA-BS in triplicate at 37°C. Standard deviation (n=3) is shown.

The results presented in Figure 2.11 indicate once again that the recovery of viable cells on BHA-BS was hindered using the neat sample when the low a<sub>w</sub> TSB was present in the suspension fluid but that this inhibition was eliminated upon removal of the low a<sub>w</sub> broth and resuspension of the cells in diluent. When the cells were resuspended in fresh broth with low a<sub>w</sub> (0.90) recovery on BHA-BS was again reduced and this was comparable to that observed when cells were suspended in the low a<sub>w</sub> TSB in which they were stressed. Resuspension in fresh TSB resulted in improved recovery of viable cells indicating that this solution was as effective as diluent in recovering cells. In contrast, resuspension in fresh MM did not allow as much recovery. Nonetheless, the number of viable cells enumerated was still greater than that observed when the suspension fluid contained salt.

## **2.4 DISCUSSION**

### **2.4.1 Assessment of Laboratory Systems Used to Describe Non-Thermal Inactivation Kinetics**

Laboratory-derived inactivation data are frequently used to judge whether a specified inimical process is likely to provide a microbiologically safe food (van Schothorst and Duke, 1984). The use of laboratory systems over direct in-product testing is preferred in some cases; to generate large amounts of data and study pure cultures, and essential in others; for example when bacteria cannot be introduced to the food processing line or when sampling directly from the food leads to low reproducibility. Consequently, much of what is known about bacterial inactivation in response to inimical processes has been derived from studies using laboratory-based systems rather than the more costly and time-consuming in-product trials.

With this said, it should be noted that there is no universal approach to the use of laboratory-based systems. Various factors, such as the preparation of the inoculum and conditions during the inimical treatment (e.g. medium type, temperature and aeration level) may vary between systems. It is widely held that any condition that influences the composition of the bacterial cell or its physiological state will affect the microbe's susceptibility to injury or death (Busta, 1978) and so such variations between laboratory systems may influence the observed level of bacterial survival. This fact prompted a series of investigations aimed at developing an appropriate and reproducible laboratory-based system for the study of *E. coli* inactivation in response to non-thermal stress.

#### **2.4.1.1 Inoculum Preparation**

*E. coli* in the stationary phase of growth develop increased resistance to a variety of environmental stresses such as starvation, high or low  $a_w$ , pH or temperature and exposure to UV radiation (Siegele and Kolter, 1992; Kolter *et al.*, 1993). Due to the induction of a large set of genes upon entry of cells into stationary phase (Hengge-Aronis, 2000), these microbes are better able to survive stress than their exponentially-growing counterparts. With this said, a number of authors (Withell, 1942; Booth, 1999; Brown, 2002) have demonstrated that even bacterial populations prepared at discrete times under standardised conditions may exhibit varying cellular physiology that can influence the behaviour of the population upon subsequent exposure to stress.

To minimise the physiological differences between microbes within stationary phase populations prepared at different times, the preparation of experimental inocula used in the current work aimed to establish a single and well-defined methodology. The method of Brown (2002) was adopted, which involved growing a  $-80^{\circ}\text{C}$  stock of *E. coli* for 14 hours at  $37^{\circ}\text{C}$  on TSA. Five of the resulting colonies are transferred to an 80 mL volume of TSB and incubated at  $37^{\circ}\text{C}$  for 24 hours, resulting in a viable count of approximately  $9.0 \log \text{CFU.mL}^{-1}$ . A modification to this method was proposed by the candidate that is less critically dependent on incubation times and thereby allows for delays during the preparation of other aspects of the experiment. The default method was modified so that colonies grown on TSA were stored at  $4^{\circ}\text{C}$  for some time period (less than 72 hours) prior to their transfer to TSB. The impact of this modification on the subsequent inactivation of stationary phase populations of *E. coli* M23 in response to low  $a_w$  and low pH was tested. Results indicated that preparation of the inoculum using either method did not influence the inactivation kinetics of *E. coli* M23 exposed to low pH. In contrast, the cold storage of *E. coli*

M23 colonies prior to their inoculation to TSB did alter the inactivation of these microbes in response to low  $a_w$ . Although the differences were not large, the same pattern was seen in two discrete experiments and suggested that the difference was reproducible and not some artefact of a single experiment.

Within the food industry meats are held at low temperature at numerous stages in the processing line. Results presented here indicate that this cold storage could lead to a slight increase in the survival of *E. coli* if later exposed to low  $a_w$  conditions. Although this phenomenon was not investigated further in the present work, it appears that the inclusion of a low temperature incubation stage during the preparation of experimental inocula may provide a laboratory system that is more relevant to the food industry.

Despite this, the method for inoculum preparation that did not include a cold-storage step was chosen as the protocol for all subsequent experiments. The more stringent method was considered most likely to provide reproducible data between temporally distinct experiments as well as to maintain consistency with other studies referred to above. The preparation of stationary phase *E. coli* populations described within the literature often use colonies that are either fresh or stored at 4°C for some amount of time. The current work indicates that this may have some influence on the non-thermal inactivation of the derived population. However, because different laboratory systems will usually contain other methodological variations, and presumably some that will be of more significance, it is unlikely that the inclusion or exclusion of low temperature incubation during inoculum preparation will invalidate any comparisons between laboratory-derived data generated with these systems. The impact of even small variations in inoculum preparation protocols on bacterial inactivation is, however, recognised.

#### **2.4.1.2 Suspension Medium for Stress Treatments**

The environment of a bacterial population during exposure to an inimical process is chiefly defined by the medium employed, which is most often one of a large number of commercially available synthetic broths. The current investigation evaluated the inactivation of *E. coli* M23 suspended in four media (TSB, NB, LB and MM-B) in response to low  $a_w$ , low pH or both. The media tested are used routinely in laboratory systems described in the literature and differ most notably in their nutrient composition, particularly complex organic molecules and metabolic 'building blocks'. Importantly, MM-B is low in pre-formed organic molecules, whereas TSB, LB and NB are all nutrient-rich, or complex, medium types.

Results presented in Section 2.3.1.2 show that the inactivation of *E. coli* M23 in response to low  $a_w$  and/or low pH treatments is highly dependent on the medium used during application of that stress. These results indicate that any inactivation data derived from studies that do not use the same suspension broth may not be comparable. In spite of this, a review of the literature shows few reports that have directly evaluated the influence of the media employed for the study of bacterial inactivation in laboratory systems.

Dealing specifically with bacterial injury, Ray and Speck (1973) have shown that the inclusion of milk, sugars and proteins in the suspending medium can greatly reduce injury in *E. coli* and other bacteria due to freezing, freeze-drying and heat treatments. Conversely, the presence of salt, acid and sanitisers can act as a secondary stress and thereby increase the level of injury observed (Ray and Speck, 1973). That work indicated that populations of *E. coli* are more resistant to stress when suspended in complex media than when suspended in minimal media. By contrast, the results of the present study illustrate that *E. coli* M23 populations

exposed to low  $a_w$  or low pH have improved survival in media lacking preformed nutrients (other than glucose) compared to that in complex broth. This implies that ingredients of complex media that aid *E. coli* cells in their resistance to freezing, freeze-drying and heat treatments may not benefit that same population during exposure to low  $a_w$  or low pH. Alternatively, some component or components within the complex media assessed in the current study may have acted as a secondary stress on osmotic or acid stressed *E. coli* M23 populations.

Some evidence for the latter possibility has been reported, although in connection with recovery media. It is generally accepted that a nutritionally rich, non-selective medium will aid the recovery of viable cells, including those injured by chemical or physical means. Nonetheless, enumeration using minimal medium can dramatically improve the observed level of recovery of stressed cells (Gomez and Sinskey, 1973; Gomez and Sinskey, 1975; Wilson and Davies, 1976; Tang and Jackson, 1979). This phenomenon has been termed the minimal medium effect and has been attributed, at least in part, to the formation of DNA lesions caused by the toxicity of ingredients in complex media (Gomez and Sinskey, 1975). If this is the case, the same may occur during suspension in complex broths, with the presence of toxic compounds within the complex media acting as a secondary stress during exposure to the inimical treatment under review.

The results of the present study are complicated by the fact that the survival of *E. coli* M23 in MM-B when exposed to low  $a_w$  or low pH is enhanced in comparison to survival in complex media. This effect is reduced, however, when exposed to these stresses simultaneously and, under these conditions, *E. coli* M23 survives better in nutrient-rich broth. In this case, the complex media may provide the bacterial population with a component that better equips them to survive the inimical

treatment. These findings suggest that the response of *E. coli* to stress in each medium is dependent not only on the type of stress imposed but also the intensity of the overall stress that the population endures.

The discussion to this point has dealt with the survival of *E. coli* in nutrient-rich media as compared to that in minimal broth. The results of the current work further indicate that the level of inactivation in *E. coli* M23 exposed to non-thermal stress in complex broth is dependent on the composition of that suspension medium. This suggests that even small differences in the nutritional composition of the environment during stress imposition can alter the survival characteristics of the bacterial population. Components that may be of influence, and have been previously shown to alter the level of recovery of stressed bacterial populations, are cations  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  (Hurst and Hughes, 1981) and  $\text{K}^{+}$  (Payne, 1978), carbohydrates (Payne, 1978), tricarboxylic acid (TCA) cycle intermediates (Hurst *et al.*, 1976; D'Aoust, 1978) and amino acids including alanine, aspartic acid, cysteine, isoleucine, serine and valine (Moss and Speck, 1966; Kuo and Macleod, 1969; Busta and Adams, 1972; Payne, 1978; Wallen and Walker, 1979; Hurst and Hughes, 1981).

It is beyond the scope of the current investigation, however, to determine experimentally which nutritional components in the suspension medium, if any, can account for the differences in survival kinetics described above. It is, however, important to select the most appropriate suspension broth for use in subsequent investigations to more accurately detail *E. coli* inactivation in response to non-thermal stress. Ideally, the suspension broth will simulate the conditions that bacteria encounter within the food matrix. Where multiple broths do this, the more appropriate suspension broth is presumably the one that allows for the best survival of *E. coli* under the specified inimical process because this is less likely to

overestimate the lethality of the inimical process compared to when the bacteria are present in foods.

The use of minimal media in laboratory systems is generally reserved to evaluate the effect of specific components on bacterial behaviour when these components are difficult to exclude from complex media. Simple defined media are not used to simulate food systems because they lack numerous components available to bacteria within most foods, such as an excess of protein and energy sources. For this reason, MM-B was not selected as the suspending medium for subsequent investigations despite the fact that for two of the three stress treatments tested the survival of *E. coli* M23 was greatest in this medium.

Of the remaining three media tested, all are complex broths and thereby simulate to a certain extent conditions encountered by *E. coli* within the food matrix. However, no single medium maximised *E. coli* M23 survival under all three treatments tested. Thus, selection of the suspension medium based on the 'best survival' criterion described above was not explicit. Instead, TSB was chosen as the primary suspension broth for subsequent evaluations of *E. coli* survival because it is used most widely in the literature and also within other related studies in this laboratory and is therefore more likely to provide valid comparisons with other data.

#### **2.4.2 Evaluation of the Diluent Used for the Enumeration of Uninjured *Escherichia coli* Exposed to Non-Thermal Stress**

Culture-based enumeration was used throughout the current study to quantify the number of *E. coli* able to survive non-thermal stresses and to evaluate the level of injury within these populations. BHA-P was used to determine the total viable



population and the selective medium BHA-BS was employed to measure the uninjured population. The bile salts in BHA-BS inhibit the growth of cells that have sustained damage to the cell membrane, to enzymes that degrade toxins and/or to efflux systems (Beuchat, 1978; Gunn, 2000). Therefore, it should be noted that using this assay the 'uninjured' population could include cells that have sustained injury to some other cellular site that does not render the cell sensitive to bile salts, such as damage to DNA molecules or impaired protein synthesis. Further, some cells may be too injured to grow even on non-selective media and, thus, would not be included in the quantitative estimate of the injured population.

The enumeration method employed involved removing a sample from the test broth and serially diluting it in diluent and plating an aliquot of the appropriate dilution(s) onto agar medium. While this method is typical of culture-based protocols employed in many laboratories, it does not give an entirely accurate account of the number of viable cells within the population at the time of sampling because it assumes that a single cell will grow to a detectable level when provided with a favourable environment. In practice, bacteria do not necessarily multiply the moment they are given the opportunity to do so but rather display a period of repair or adjustment to the new environment prior to division, which is termed the division lag (Postgate, 1967). Consequently, if a bacterial cell is determined to be non-viable by the culture-based methodology described above it remains unclear whether it was killed by the inimical process *per se* or during the division lag, which commences upon removal of the sample from the original population. As a result, culture-based determination of the viability of a bacterial population is actually a measure of the viability at the time of sampling minus the inactivation that occurred during the division lag. Equally, a measure of the level of injury in a population of

*E. coli* includes the level of injury at the time of sampling plus any further injury that occurs during the division lag.

It is important that enumeration methodologies attempt to minimise the level of mortality or injury that occurs during the division lag to give a more accurate account of the level of viability or injury within the population of interest. This is particularly crucial during the enumeration of populations exposed to inimical conditions because a proportion of the viable cells will have sustained injury and are thereby rendered more susceptible to secondary stresses (Mackey, 2000). Should the enumeration procedure expose these cells in particular, but essentially any viable cell to a secondary stress, they may become inactivated or their growth inhibited and the efficacy of the inimical treatment will be overestimated.

Brown (2002) showed that recovery of *E. coli* following exposure to non-thermal stresses was optimised by enumeration on BHA-P. However, the suitability of the same enumeration protocol to quantify the uninjured population of *E. coli* on BHA-BS required further evaluation. Initial experiments conducted as part of this evaluation indicated that the recovery of uninjured cells on BHA-BS was hindered when plating undiluted samples from populations exposed to low  $a_w$ . This trend was not observed in populations exposed to low pH conditions and suggested that the increased salt concentration in the low  $a_w$  TSB caused the reduced recovery of cells on BHA-BS. It has been reported that NaCl may act as a selective agent when included in the recovery medium (Scheusner *et al.*, 1971) and, thus, an increased level of salt in the suspension fluid and its transfer to BHA-BS may have inhibited the recovery of *E. coli* M23 that were not sensitive to bile salts. Alternatively, the higher concentration of salt on BHA-BS may have damaged cells that were uninjured at the time of sampling and caused them to become sensitive to bile salts.

The same effect due to increased levels of hydrogen ions in the low pH samples may have been ameliorated by the buffering capacity of the media.

Removal of the high salt medium from the neat sample by centrifugation and resuspension of the cells in diluent was able to reverse the low recovery level of uninjured cells described above. The process of removing the suspension fluid from diluted samples of the stressed population caused a slight loss in the recovery of viable cells, probably due to experimental error (e.g. all the cells may not have been pelleted, some were lost when removing the supernatant or additional cell killing or injury may have occurred prior to the removal of the low  $a_w$  broth). Although this loss was very small, it was decided that the removal of the low  $a_w$  medium from the suspension fluid was necessary to reduce experimental error only when using neat samples. Figure 2.9 highlights the usefulness of incorporating this step when uninjured cells are in low numbers (i.e. less than  $4.0 \log \text{CFU.mL}^{-1}$ ) and maintains that if the treatment medium is not removed prior to plating it is likely that the level of injury in *E. coli* exposed to low  $a_w$  will be overstated. Consequently, the protocol employed for the enumeration of uninjured *E. coli* cells on BHA-BS subsequently included removal of the high salt TSB from neat samples by the method described. Inclusion of this step when *E. coli* M23 populations were exposed to low  $a_w$  in MM-B was deemed to be unnecessary because the recovery of uninjured cells on neat plates was not reduced in this instance.

That the recovery of *E. coli* M23 treated with low  $a_w$  was inhibited on BHA-BS in the presence of high salt and TSB but not high salt and MM-B suggested that the recovery inhibition may have been due to the combination of high salt and some component present in TSB but not MM-B. This putative component could be an ingredient of TSB or could have been produced or modified by *E. coli* M23 during

exposure to the osmotic stress. In the latter case, the production or modification of that component presumably occurred in TSB but not MM-B because of physiological differences between the cells or because essential substrates were not available in the minimal medium.

Investigations to test the above hypothesis indicated that the recovery of cells treated with low  $a_w$  (in TSB) on BHA-BS was equally reduced when using any of the three high-salt suspension fluids (low  $a_w$  TSB in which the cells were treated, or freshly prepared MM and TSB with low  $a_w$ ). These findings suggested that the recovery of low- $a_w$  stressed *E. coli* M23 on BHA-BS was not inhibited by (a) a component included as an ingredient in TSB but not MM-B; and (b) a modification to the suspension broth during the application of low  $a_w$  in TSB. Importantly, the use of TSB without added salt as the suspension fluid showed no reduction in the recovery of uninjured *E. coli* M23 and it appeared that the high levels of salt within the suspension fluid could, on its own, account for the reduction in the level of recovery of osmotically stressed *E. coli* M23 on BHA-BS. That a high salt suspension fluid was inhibitory to the recovery of uninjured cells when *E. coli* M23 was exposed to low  $a_w$  in TSB but not in MM-B suggested that (a) some property of MM-B enhanced recovery on BHA-BS or (b) some physiological difference between these populations existed. The latter further emphasises the impact of the environment during application of non-thermal stress on the response of *E. coli* as previously described in Section 2.4.1.2.

### **2.4.3 Conclusions**

The inactivation and injury observed in bacterial populations exposed to inimical conditions can vary substantially according to the materials and procedures that are

used to enumerate cells. In this study, the consequence of making even slight adjustments in the preparation of the inoculum was highlighted. Further, the environment of the microbial population dramatically influenced the survival characteristics of *E. coli* M23 exposed to non-thermal challenges. Since no single medium (LB, NB, MM-B or TSB) ensured the best survival of *E. coli* M23 in response to all three treatments ( $a_w$  0.90, pH 3.5 or both), it appears that bacteria differ in their survival characteristics depending on the type and intensity of the total stress applied. Although the use of the culture media that is the most often utilised in inactivation trials allows for valid comparisons of results, selection of media based on empirical evaluations of their effects may be more suitable. It was also shown that typical enumeration procedures might not be optimal for the recovery of the uninjured proportion of low  $a_w$ -treated *E. coli* M23 populations. A proper determination of the level of injury in these populations requires that exposure to NaCl is minimised during the enumeration procedure. This work provides a means to select appropriate materials and methods to better define bacterial viability and injury in response to inimical processes. Investigations of the effect of low  $a_w$  on *E. coli* presented in later chapters drew on findings presented here.

## Chapter 3

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# **Characterising Viability and Injury in *Escherichia coli* in Response to Non-Thermal Stress**

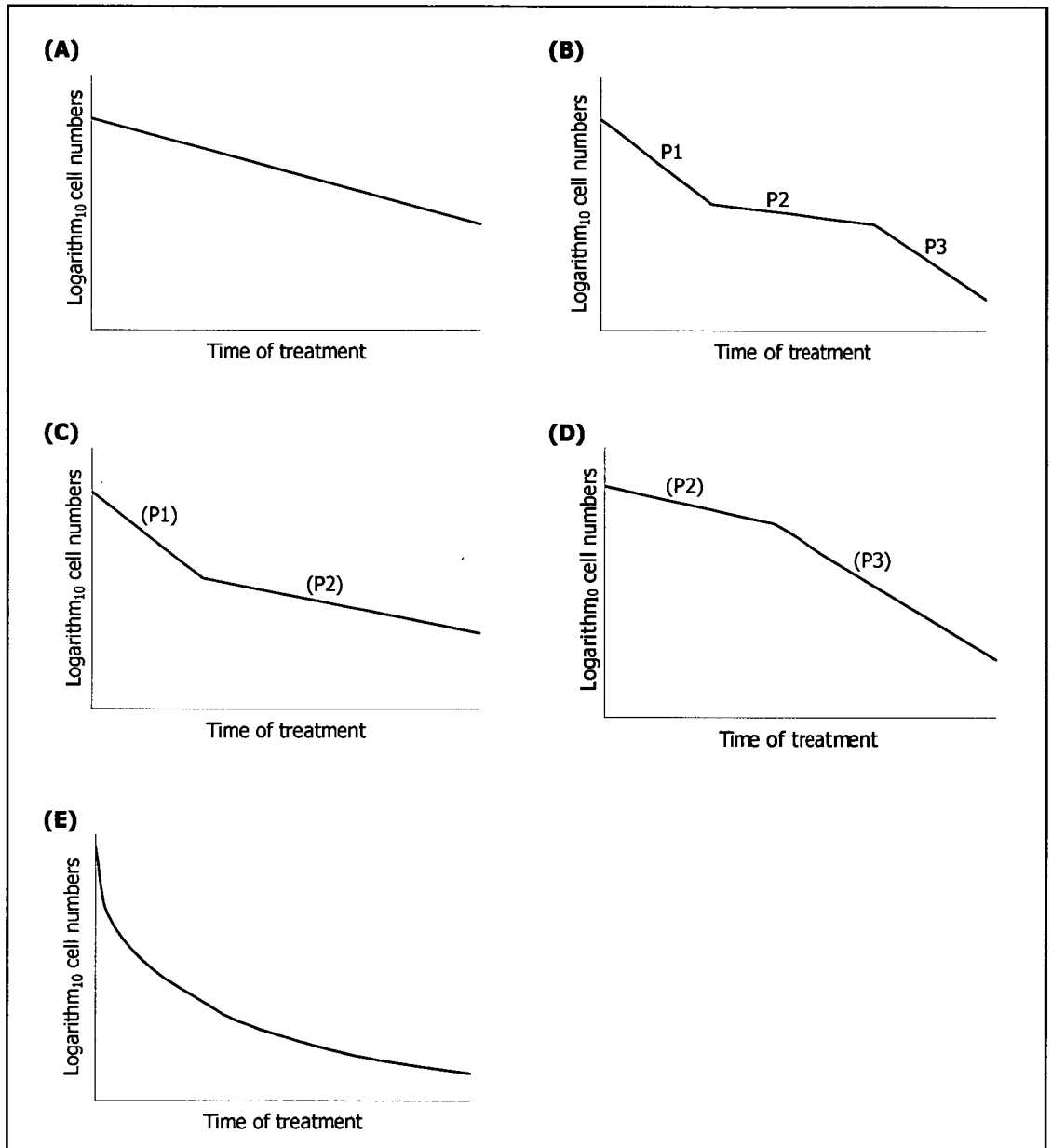
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### 3.1 INTRODUCTION

The kinetics of microbial inactivation were described in the early 1900's and demonstrated that the  $\log_{10}$  of the number of viable cells exposed to an inimical environment declined linearly as a function of time (Madsen and Nyman, 1907 cited by Withell, 1942). This phenomenon was initially described in bacterial populations exposed to heat (Chick, 1910), disinfectants (Madsen and Nyman, 1907 cited by Withell, 1942; Chick, 1908; Chick, 1910) and other lethal agents (Paul, 1908; Lee and Gilbert, 1918; Wyckoff, 1930) and suggested that log-linear inactivation could be universally applied to the death of microbial populations in response to any environmental parameter. Described mathematically as  $N_t = N_0 e^{-kt}$  (where  $N$  is the number of survivors at time  $t$  from an initial number  $N_0$  and  $k$  is a rate constant), bacterial death was interpreted to be a first-order reaction caused by the inactivation of one critical site per cell (Madsen and Nyman, 1907 cited by Humpheson *et al.*, 1998). Consequently, data concerning the loss of viability in bacterial populations exposed to lethal agents are represented graphically as 'survival curves', i.e. a plot of the  $\log_{10}$  of viable numbers against time of treatment.

Further investigations on the loss of viability in bacterial populations reported deviations from the above-described log-linear relationship (Rahn, 1930; Moats *et al.*, 1971; Cerf, 1977). Including biphasic (with a 'shoulder' or 'tail'), curvilinear (concave or convex), and other, more complex deviations, the current literature is filled with reports of survival curves that include portions of log-linearity but which are complicated by departures from the law of log-linear decline (Whiting, 1993; Brown *et al.*, 1997; Gustafson *et al.*, 1998; Humpheson *et al.*, 1998; Shadbolt *et al.*,

1999; Uyttendaele *et al.*, 2001). Examples of the more common forms of deviations are shown in Figure 3.1.



**Figure 3.1** Common types of survival curves. A log-linear inactivation; B triphasic inactivation characterised by Phase 1 (P1), Phase 2 (P2) and Phase 3 (P3); C biphasic inactivation including 'tailing' and containing phases analogous to P1 and P2; D biphasic inactivation with an initial 'shoulder' and, therefore, phases similar to P2 and P3; and E concave inactivation (see text for details).

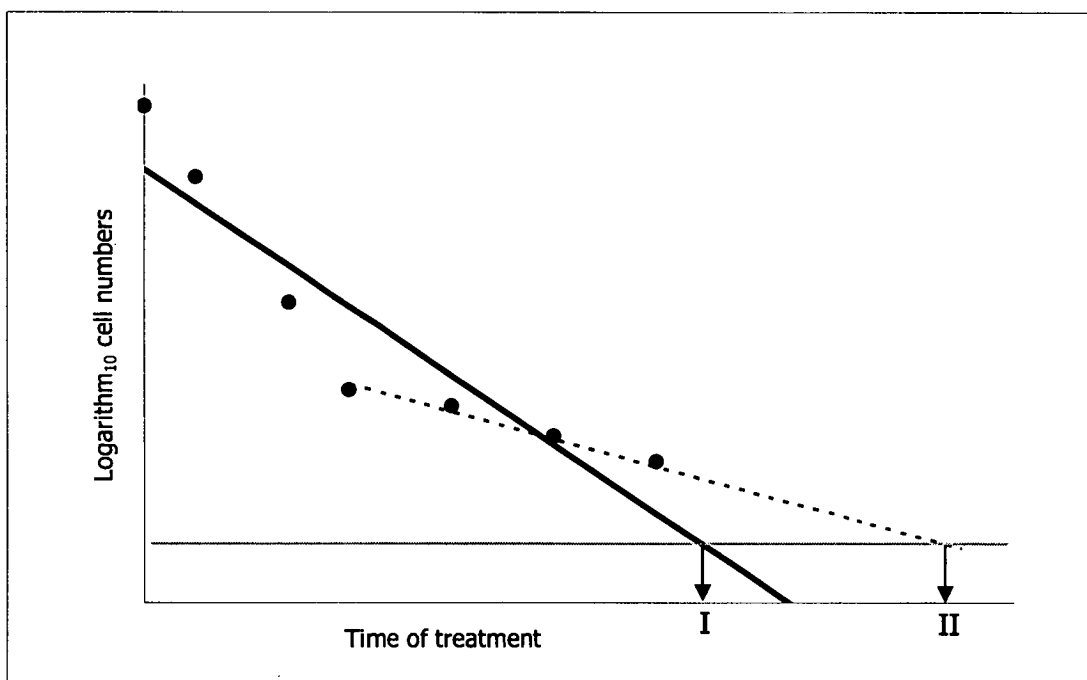


Depicted in Figure 3.1 (B) is a triphasic inactivation curve that has been observed by Brown (2002) in *E. coli* populations exposed to low pH. In that instance, three distinct phases of log-linear decline (Phase 1, P1; Phase 2, P2; and Phase 3, P3) were evident. In those analyses, P1 is considered as an initial and relatively rapid phase of inactivation that is followed by a slower rate of decline (P2) and then a final phase of inactivation (P3) that exhibits a rapid rate and occurs until viable cells cannot be detected by culture-based enumeration. It is possible that experiments that reveal only biphasic survival curves fail to detect either P1 or P3.

Many authors have attempted to explain the basis of the characteristics of non-log-linear survival curves (Lee and Gilbert, 1918; Stumbo, 1965 cited by Tomlins, 1976; Moats *et al.*, 1971; Cerf, 1977). In general, concave curves are considered to arise from populations that are heterogeneous and as such the cells exhibit different levels of tolerance to the inimical process (Lee and Gilbert, 1918). Shoulders or tails are the slower phase of inactivation in curves that exhibit two distinct processes of log-linear decline. Shoulders are considered to indicate that each cell requires 'multiple hits' before it is inactivated and therefore there exists an initial lag prior to bacterial death (Moats *et al.*, 1971). Conversely, shoulders may be caused by excessive cell clumping, which initially protects a proportion of the population from the lethal agent (Stumbo, 1965 cited by Tomlins, 1976). Tailing may arise from experimental artefacts, for example due to faulty testing procedures or equipment, or as a result of clumping of dead cells around those still viable that affords them protection (Cerf, 1977). Otherwise, the existence of tails might be a result of inherent variability within the population that is demonstrated by the appearance of a subpopulation of resistant cells, or due to cells adapting to the stressful environment and being able to survive the inimical process for longer (Moats *et al.*, 1971; Cerf, 1977).

Except for some early studies reviewed by Cerf (1977), the majority of evidence suggests that experimental practices cannot account for the appearance of multiphasic survival curves. Using procedures that minimise experimental artefacts including cell clumping (Shadbolt, 2004), protective effects from dead cells (Rahn, 1943; Moats *et al.*, 1971; Humpheson *et al.*, 1998) and enumeration-related limitations (Humpheson *et al.*, 1998), such factors have been excluded as causes of non-log-linearity in survival curves. Those investigations have confirmed that the non-log-linear loss of viability of bacterial populations is a real phenomenon that can be modified by experimental practices but are not, in themselves, artefacts.

The emergence of deviations from log-linear decline in bacterial populations treated with an inimical stress has practical implications. Traditionally, the food industry has used the concept of log-linear inactivation in response to a given stress to estimate the time required to reduce the initial bacterial load to a level that is safe for consumption (Cerf, 1977). Because this approach relies on extrapolation, it is obviously flawed where deviations from log-linear decline exist. As shown in Figure 3.2, extrapolation of the linear regression equation fitted to non-linear (in this case, biphasic) data can give a poor estimation of the time required to reduce the bacterial population to safe levels. Therefore, prior to applying such methods in food microbiology for production of safe processing conditions it is crucial to recognise variations from log-linear inactivation. Importantly, there appears to be no consistent association between the observed deviations from log-linear inactivation and the bacterial species or lethal agents involved and thus the kinetics of inactivation of a pathogen in response to a specific stress needs to be determined empirically. This is true for low  $a_w$ -treated *E. coli*.



**Figure 3.2** The non-linear decline of a hypothetical bacterial population exposed to a particular stress. The black, solid line is a linear regression fitted to the data. Extrapolation of the linear regression indicates that the population will reach a level that is safe for consumption (corresponding to the grey line) at Point I. However, if the population continues to decline at the second, slower rate of inactivation (given by the dashed line) then the population will not reach safe levels until Point II (see text for details).

Outbreaks of disease caused by EHEC and linked to the consumption of foods characterised by low  $a_w$  and/or low pH have demonstrated the ability of *E. coli* to survive these environments (CDC, 1995a; CDC, 1995b; Bettelheim, 2000; MacDonald *et al.*, 2004). The endurance of viable *E. coli* cells after prolonged exposure to low  $a_w$  in laboratory systems has further established the potential of *E. coli* to survive osmotic challenges relevant to food manufacture (Clavero and Beuchat, 1996; Shadbolt *et al.*, 1999). Coupled with the low infective dose of pathogenic strains of *E. coli* (Tilden *et al.*, 1996), this tolerance to low  $a_w$  is of great concern to the food industry. Very few investigations pertaining to the loss in viability in populations of *E. coli* exposed to low  $a_w$  environments are available in the

literature. As a result, knowledge of the kinetics of low  $a_w$ -induced inactivation of *E. coli* is lacking.

The studies described in this chapter aim to characterise the kinetics of inactivation of *E. coli* in response to low  $a_w$  environments. Given that food systems often utilise other inimical processes in conjunction with low  $a_w$  to achieve a microbiologically safe product, the death of *E. coli* following exposure to low  $a_w$  in combination with low pH and the order in which those stresses are applied was examined. Moreover, this work attempted to gain insights into the mechanisms of inactivation of *E. coli* in response to non-thermal stress environments via evaluation of the level of injury.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Bacterial Strains, Reagents, Media, Solutions and Equipment**

Details of bacterial strains, chemical reagents, bacteriological media, solutions and equipment (including software) are given in Appendix A. All experiments described in this chapter employed the non-pathogenic *E. coli* M23.

### **3.2.2 Analysis of the Kinetics of Low Water Activity-Induced Inactivation of *Escherichia coli***

Stationary phase populations (2.2.2.1) of *E. coli* M23 were exposed to low  $a_w$  in TSB [ $a_w$  0.90, 25°C, TSB] (2.2.2.3) in two separate experiments. Immediately prior to the treatment, and at regular intervals throughout, the number of viable cells was determined by culture-based methods (2.2.2.4).

### **3.2.3 Evaluation of the Inactivation of *Escherichia coli* in Response to Low Water Activity in Combination with Low pH**

#### **3.2.3.1 Simultaneous Application of Osmotic and Acid Stresses**

Stationary phase populations (2.2.2.1) of *E. coli* M23 in TSB were exposed to low  $a_w$  [ $a_w$  0.90, 25°C, TSB], low pH [pH 3.5, 25°C, TSB], or low  $a_w$  and pH [ $a_w$  0.90, pH 3.5, 25°C, TSB] (2.2.2.3). The number of viable cells was estimated by culture-based enumeration (2.2.2.4) immediately prior to, and at regular intervals throughout the treatment.

#### **3.2.3.2 Order of Application of Osmotic and Acid Stresses**

Due to the reduced buffering capacity of MM-B, addition of NaCl to that medium causes a greater decline in pH than it does in TSB. To assess the effect of pH changes caused by the addition of NaCl on *E. coli* inactivation the current experiment, therefore, used MM-B. A stationary phase population (2.2.2.1) of *E. coli* M23 was exposed to a first lethal stress (FLS) of low  $a_w$  in MM-B [ $a_w$  0.90, 25°C, MM-B] (2.2.2.3). Following 24 hours of treatment, a second lethal stress (SLS) was applied by adjusting the pH to 3.50 ( $\pm$  0.05) by the addition of 10 M HCl [ $a_w$  0.90  $\rightarrow$  pH 3.5', 25°C, MM-B]. A low  $a_w$  control was prepared similarly but no SLS was imposed [ $a_w$  0.90, 25°C, MM-B].

Two stationary phase populations (2.2.2.1) of *E. coli* M23 were treated with low pH in MM-B [pH 3.5, 25°C, MM-B] (2.2.2.3) as a FLS. After 24 hours of treatment, a SLS was added by the addition of a measured amount (typically 6-8 g) of UV-sterilised NaCl to obtain a final  $a_w$  0.900 ( $\pm$  0.003) [pH 3.5  $\rightarrow$   $a_w$  0.90 with pH 2.9', 25°C, MM-B]. The addition of NaCl caused a decrease in pH of the broths and, thus, this was readjusted to pH 3.50 ( $\pm$  0.05) using 10 M NaOH for one of the

populations [pH 3.5 →  $a_w$  0.90 with pH 3.5', 25°C, MM-B], with the other treatment being left at the further reduced pH. A low pH control was prepared similarly but no SLS was imposed [pH 3.5, 25°C, MM-B].

An additional stationary phase population (2.2.2.1) of *E. coli* M23 was exposed to low  $a_w$  and low pH simultaneously in MM-B [ $a_w$  0.90, pH 3.5, 25°C, MM-B] (2.2.2.3), with no SLS imposed, as a further control. Immediately following the imposition of a first or second lethal stress and at the end of the treatment, a 2 mL aliquot was withdrawn for  $a_w$  and pH determination. Immediately prior to, and at regular intervals throughout the treatment, the number of viable cells was determined by culture-based enumeration (2.2.2.4).

### **3.2.4 Examination of Injury in *Escherichia coli* in Response to Osmotic and/or Acid Stress**

Stationary phase populations (2.2.2.1) of *E. coli* M23 in TSB were exposed to low  $a_w$  [ $a_w$  0.90, 25°C, TSB], low pH [pH 3.5, 25°C, TSB], or low  $a_w$  and pH [ $a_w$  0.90, pH 3.5, 25°C, TSB] (2.2.2.3). Immediately prior to the treatment, and at regular intervals throughout, the number of viable cells and uninjured cells were determined by plating to BHA-P (2.2.2.4) and BHA-BS, respectively.

It is a widely accepted hypothesis that bacteria are injured when they grow in a non-selective but not in a selective medium such as those containing the surface active agent bile salts (Beuchat, 1978), as described in Section 2.1. To enumerate uninjured cells (or more specifically, those that had not sustained damage to the outer membrane, enzymes that degrade bile salts and/or efflux systems that export bile salts from the cell), aliquots (100  $\mu$ L) were withdrawn from each population,

serially diluted in diluent and 50 or 250  $\mu\text{L}$  volumes surface plated to BHA-BS. Where neat samples contained low  $a_w$  TSB, the broth was removed by centrifugation at 10 000 rpm for 10 minutes at  $4^\circ\text{C}$  in a Microcentrifuge 5417R and the pellet resuspended in an equal volume of diluent prior to plating to BHA-BS. Plates were incubated at  $37^\circ\text{C}$  for  $14 (\pm 0.5)$  hours and CFU recovered on each medium type enumerated using an image scanner and analysis software. Injured profiles were constructed by plotting  $\log \text{CFU.mL}^{-1}$  against time. The minimum level of detection using this method is detailed in Section 2.2.2.4.

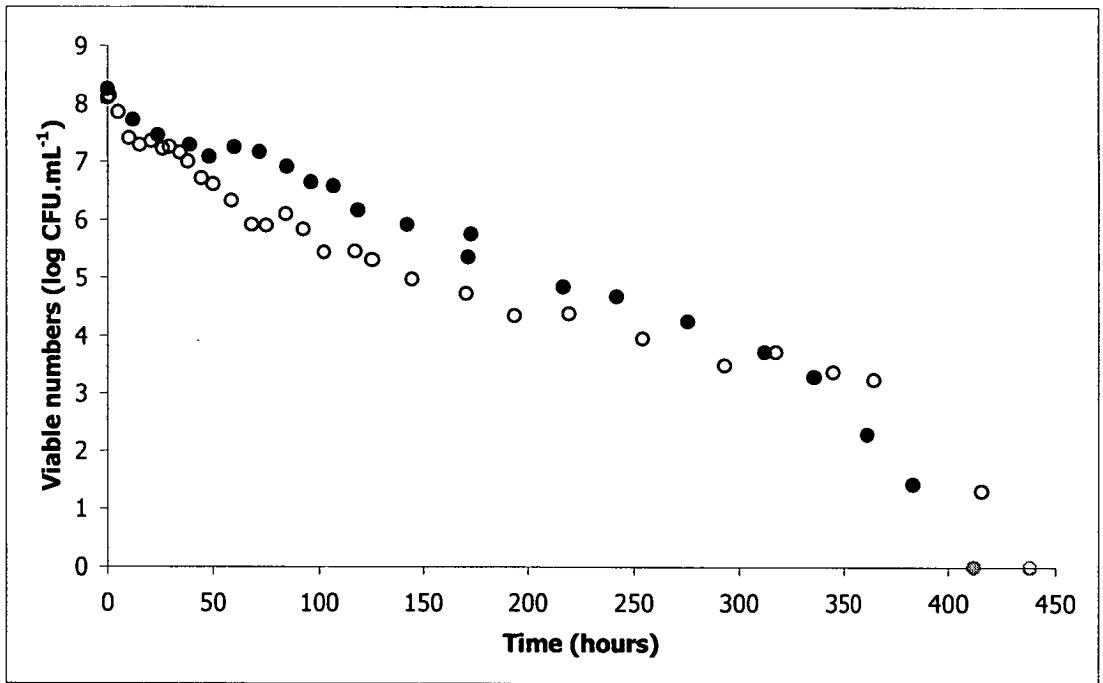
Differences in the absolute number of cells recovered on BHA-P and BHA-BS were regarded as the proportion of the population that had sustained injury (limited to those cellular components mentioned above). The percent-injured population at each time point was calculated using the absolute number of cells as:

$$\text{Percent injury} = 100 \times \left( \frac{(\text{Cells on BHA-P}) - (\text{Cells on BHA-BS})}{(\text{Cells on BHA-P})} \right)$$

### 3.3 RESULTS

#### 3.3.1 Analysis of the Kinetics of Low Water Activity-Induced Inactivation of *Escherichia coli*

Initial investigations attempted to characterise the time-dependent loss of viability in populations of *E. coli* exposed to low  $a_w$  and, further, to determine the inherent variability between temporally distinct experiments that used identical methodology. Depicted in Figure 3.3 are the inactivation kinetics of stationary phase populations of *E. coli* M23 exposed to low  $a_w$  [ $a_w$  0.90,  $25^\circ\text{C}$ , TSB] as determined over two temporally separate experiments (designated Populations A and B).



**Figure 3.3** The inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 (NaCl as humectant) at 25°C in TSB in two separate experiments (● Population A, and ○ Population B). Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

The inactivation rate of Population A was nearly constant for the first 150 hours of treatment with low  $a_w$ , although this included a slight decrease in the rate of inactivation at around 50 hours that shifted the inactivation profile upwards. The inactivation of the population appeared to occur at a slightly slower rate from around 150 to 300 hours. The rate of inactivation then increased and this more rapid decline continued until viable cells were below the minimum level of detection (i.e. undetected when plating a 250  $\mu$ L volume to BHA-P and therefore plotted as 0 log CFU.mL<sup>-1</sup>) after 384 hours. Therefore, three phases were apparent during the inactivation of Population A and appear to be equivalent to P1, P2 and P3 described in Section 3.1 and reported by Brown (2002).

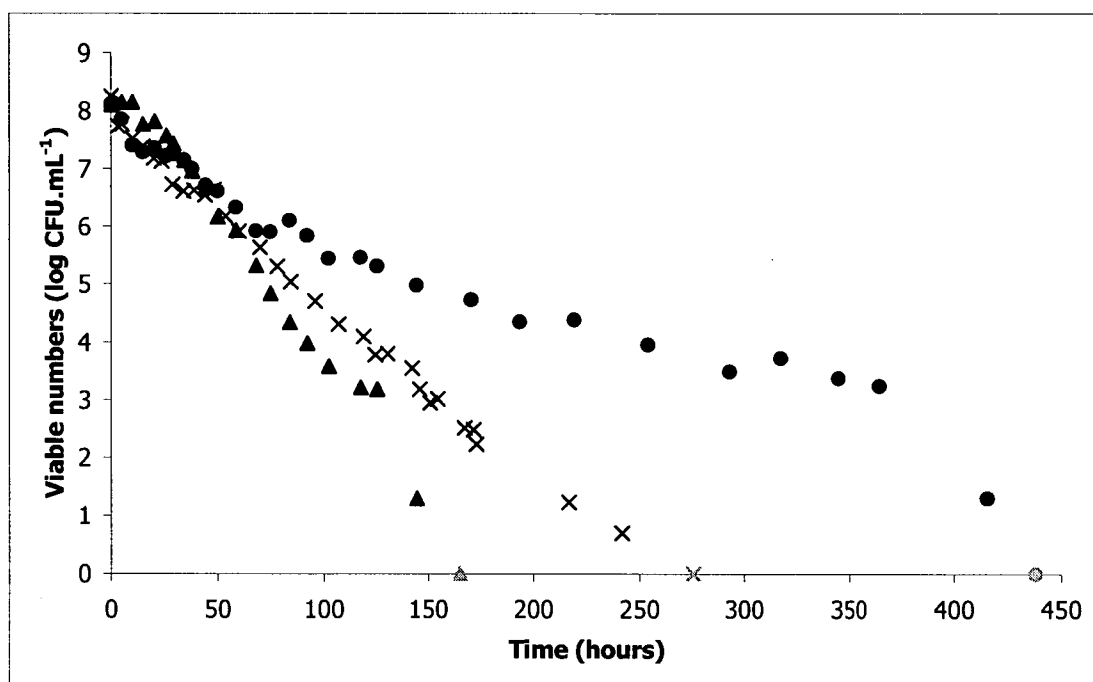


The inactivation of Population B also involved three phases, however, the duration and rate of decline for each phase differed slightly from that observed in Population A. Population B initially declined at a constant rate until approximately 150 hours of treatment with low  $a_w$ . Following this, inactivation occurred at a slightly slower rate until 350 hours and this rate was somewhat slower than the analogous second phase of inactivation of Population A. After 350 hours of treatment, the rate of inactivation of Population B increased and viable numbers declined to below detectable levels by 438 hours. Despite a small amount of variance between the separate experiments, it is apparent that an initial viable population of approximately  $8.2 \log \text{CFU.mL}^{-1}$  declines to less than  $1.0 \log \text{CFU.mL}^{-1}$  within 450 hours of treatment with  $a_w$  0.90 at  $25^\circ\text{C}$  in TSB. Inactivation appears to involve three distinct phases of inactivation.

### **3.3.2 Evaluation of the Inactivation of *Escherichia coli* in Response to Low Water Activity and in Combination with Low pH**

#### **3.3.2.1 Simultaneous Application of Osmotic and Acid Stresses**

Illustrated in Figure 3.4 is the time-dependent loss of viability of stationary phase *E. coli* M23 exposed to low  $a_w$  and low pH simultaneously [ $a_w$  0.90, pH 3.5,  $25^\circ\text{C}$ , TSB]. The inactivation of stationary phase populations of *E. coli* M23 in response to the individual stresses (i.e. low  $a_w$  [ $a_w$  0.90,  $25^\circ\text{C}$ , TSB] or low pH [pH 3.5,  $25^\circ\text{C}$ , TSB]) is also shown.



**Figure 3.4** Inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 (●), pH 3.5 (▲) or the simultaneous exposure to  $a_w$  0.90 and pH 3.5 (×) at 25°C in TSB. Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours. NaCl was used as the humectant and HCl as the acidulant.

The inactivation of *E. coli* M23 in response to  $a_w$  0.90 was described previously (i.e. data previously given in Section 3.3.1; Figure 3.3; Population B). The inactivation of *E. coli* M23 due to pH 3.5 was minimal within the first 10 hours of treatment but thereafter occurred at an approximately constant rate. Viable cells could not be detected in this population when a 250  $\mu$ L volume was plated to BHA-P (plotted as 0 log CFU.mL<sup>-1</sup>) by 165 hours. The simultaneous exposure of *E. coli* M23 to  $a_w$  0.90 and pH 3.5 exhibited single phase inactivation kinetics and caused the inactivation of the population to below the lower level of detection using a 250  $\mu$ L volume within 276 hours.

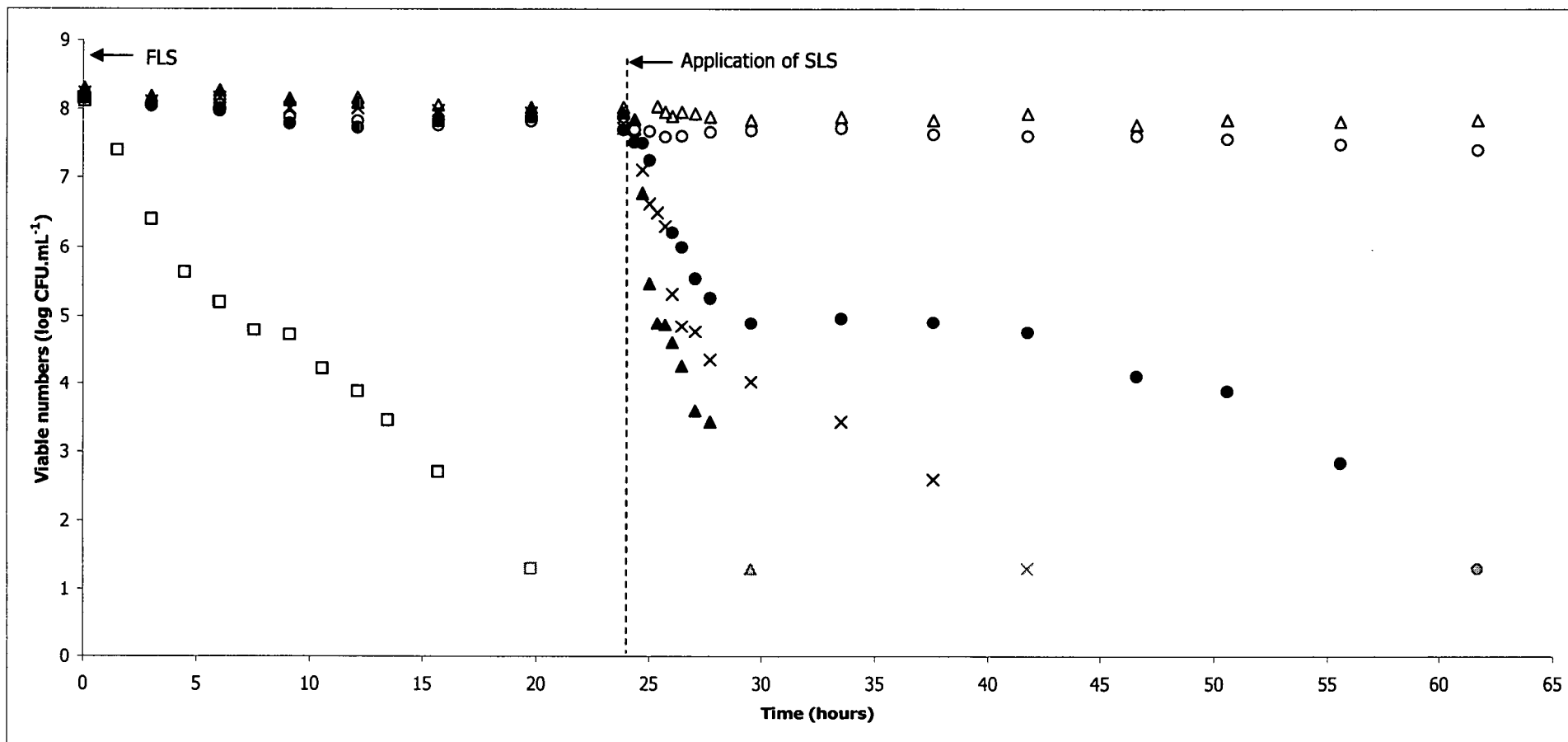
In the experiment depicted in Figure 3.4, *E. coli* M23 survived the combined low  $a_w$  and low pH treatment for longer than the individual pH stress, however, this

observation relies upon only two enumerations of the population exposed to the simultaneous osmotic and acid stress (following approximately 145 and 165 hours of treatment) when the population density was  $< 2.0 \log \text{CFU.mL}^{-1}$  and, thus, might not be reliable.

### **3.3.2.2 Order of Application of Osmotic and Acid Stresses**

The effect of the order in which low  $a_w$  or low pH stresses are applied to populations of *E. coli* M23 was assessed and is shown in Figure 3.5, which is representative of two separate experiments. It should be noted that, in these experiments, treatments were in MM-B, rather than TSB that was used in the experiments described above, because its reduced buffering capacity allowed the effect of pH changes following the addition of NaCl to be clearly defined.

Control populations experienced slight inactivation following imposition of the FLS at the commencement of the experiment. Low pH-treated [pH 3.5, 25°C, MM-B] and low  $a_w$ -treated [ $a_w$  0.90, 25°C, MM-B] populations showed relatively slow rates of inactivation. In contrast, the *E. coli* M23 population exposed to a simultaneous low  $a_w$  and low pH stress [ $a_w$  0.90, pH 3.5, 25°C, MM-B] declined rapidly and viable cells were not detected ( $< 1.30 \log \text{CFU.mL}^{-1}$ ) after 16 hours. Prior to application of the SLS all populations exhibited very similar inactivation kinetics to that of their corresponding control.



**Figure 3.5** Inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 (○; NaCl as humectant), pH 3.5 (△; HCl as acidulant) or  $a_w$  0.90 and pH 3.5 (□) at 25°C in MM-B. A SLS was added after 24 hours (●  $a_w \rightarrow$  pH; × pH  $\rightarrow$   $a_w$  with pH 3.5; ▲ pH  $\rightarrow$   $a_w$  with pH 2.9). Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

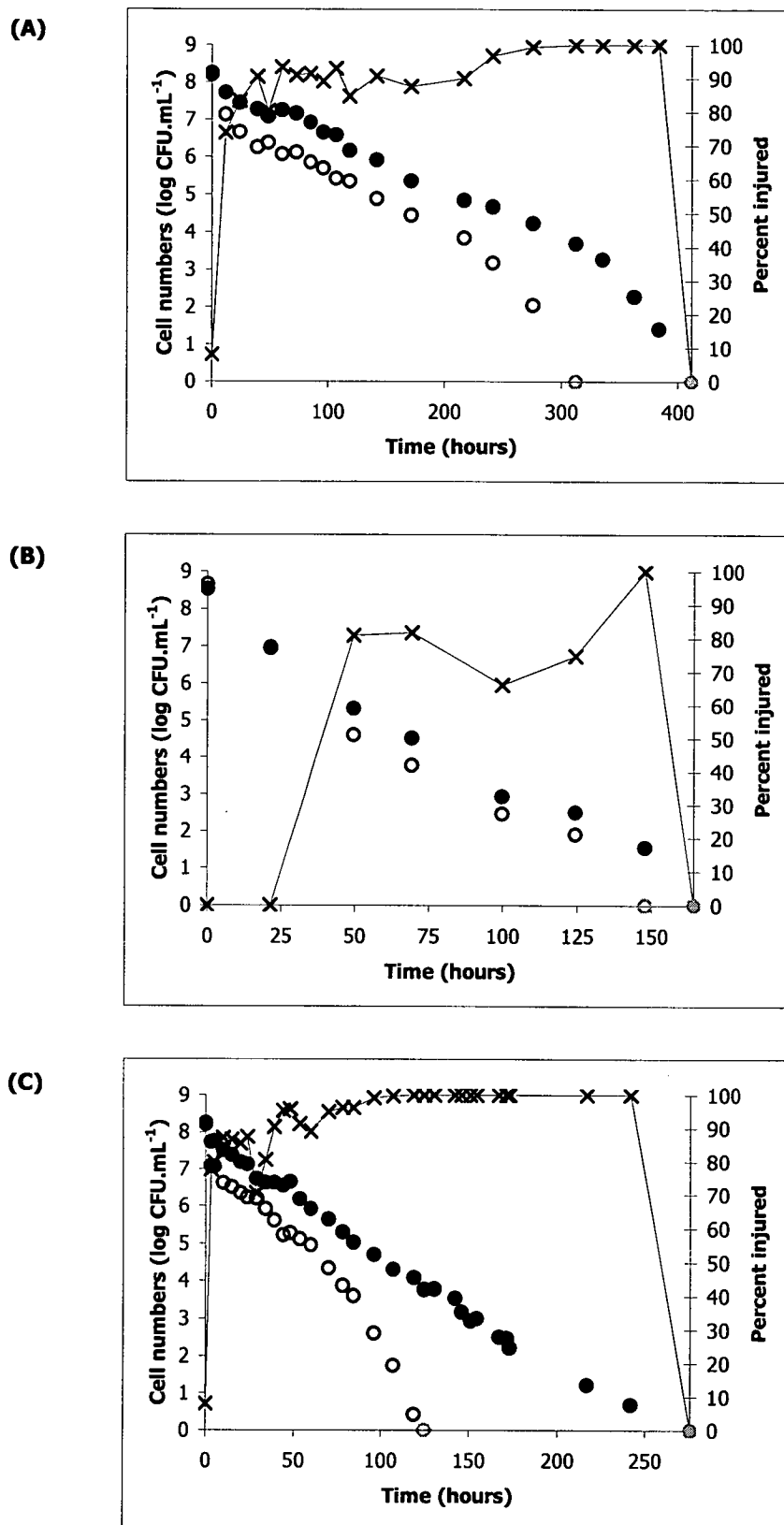
After 24 hours a SLS was applied. Two populations of *E. coli* M23 treated with low pH as a FLS were exposed to low  $a_w$  as a SLS. Alteration in the pH of the medium due to the addition of NaCl was left unadjusted [pH 3.5  $\rightarrow$   $a_w$  0.90 with pH 2.9, 25°C, MM-B] or adjusted with NaOH to pH 3.5 [pH 3.5  $\rightarrow$   $a_w$  0.90 with pH 3.5, 25°C, MM-B]. Both of these populations experienced a rapid loss in viable cells immediately following imposition of the SLS. Inactivation of the population treated with the lower pH occurred at a fairly constant rate and viable numbers were below the limit of detection within 6 hours of the SLS. In contrast, a subpopulation emerged (evidenced by a slightly slower rate of inactivation) following the initial rapid decline when the pH was readjusted to 3.5 and viable cells were detected in this treatment for at least 13 hours following the SLS.

When the population initially exposed to low  $a_w$  was further exposed to low pH as a SLS [ $a_w$  0.90  $\rightarrow$  pH 3.5, 25°C, MM-B] the rate of inactivation increased and the population declined by approximately 3 log CFU.mL<sup>-1</sup> within 5.5 hours. This was followed by the emergence of a subpopulation that then experienced two phases of inactivation. The former ('shoulder') occurred at a slower rate and lasted for about 15 hours whereas the latter phase reduced the population by about 3 log CFU.mL<sup>-1</sup> over a 20-hour period. Interestingly, a similar shoulder was evident (i.e. at approximately 5 log CFU.mL<sup>-1</sup>) in the simultaneous  $a_w$  and pH stress and in the pH  $\rightarrow$   $a_w$  with pH 3.5 treatment but was not evident in the pH  $\rightarrow$   $a_w$  with pH 2.9 treatment, which was much more rapidly inactivated than all other treatments. It appears, therefore, that populations of *E. coli* M23 exposed to a combined  $a_w$  0.90 and pH 3.5 stress, whether applied simultaneously or as a first and second lethal stress, exhibit a relatively slower phase of inactivation (or shoulder) when the population density is around 3.5 – 5.5 log CFU.mL<sup>-1</sup>.

### 3.3.3 Examination of Injury in *Escherichia coli* in Response to Osmotic and/or Acid Stress

The level of injury in populations of *E. coli* M23 exposed to low  $a_w$  [ $a_w$  0.90, 25°C, TSB], low pH [pH 3.5, 25°C, TSB] or low  $a_w$  and pH [ $a_w$  0.90, pH 3.5, 25°C, TSB] was determined by differential plating on BHA-P (viable cells) and BHA-BS (uninjured, viable cells only) and is shown in Figure 3.6, which is representative of two separate experiments. Analysis of these results suggests that osmotic shock or that in combination with acid stress causes immediate injury (as measured by bile salt sensitivity) to a large proportion of the viable population. In response to both of these treatments, the level of injury tended to increase with treatment time until at some point (313 hours for the low  $a_w$  treatment or 125 hours for the simultaneous low  $a_w$  and low pH treatment) the entire viable population was injured (i.e. cells were not recovered on BHA-BS using a 250  $\mu$ L aliquot of the population). This suggests that injury as determined by BHA-BS enumeration is not necessarily lethal in *E. coli* under inimical  $a_w$  conditions, or that in combination with low pH.

In contrast, low pH-treated *E. coli* M23 showed a markedly different injury profile. Initially, the viable population was reduced by 1.7 log CFU.mL<sup>-1</sup> within 22 hours but no bile salt sensitivity was evident. This indicates that pH-induced damage to the membrane, enzymes or efflux systems in *E. coli* is lethal or that pH does not cause injury to these sites and cell death at this stage results from irreparable damage to some other cellular components. Less than 85% of the viable population were sensitive to bile salts throughout the low pH-treatment except at 148 hours. At this time, no cells were recovered on BHA-BS from a 250  $\mu$ L volume removed from the population and, thus, > 99% of the viable population was inferred to have sustained injury to the cell membrane, to toxin-degrading enzymes and/or to efflux systems.



**Figure 3.6** Number of viable (●) and uninjured (○) *E. coli* M23 and the percent injured viable population (x; based on absolute numbers) in response to (A)  $a_w$  0.90, (B) pH 3.50 or (C)  $a_w$  0.90 and pH 3.5 at 25°C in TSB. Grey data points indicate that the number of cells was below the minimum level of detection. Viable cells were enumerated on BHA-P and uninjured cells on BHA-BS at 37°C for 14 hours. NaCl was used as the humectant and HCl as the acidulant.

### 3.4 DISCUSSION

#### 3.4.1 Analysis of the Kinetics of Low Water Activity-Induced Inactivation of *Escherichia coli* M23

The inactivation kinetics of stationary phase populations of *E. coli* M23 in response to low  $a_w$  described here exhibited deviations from log-linear decline. Three phases of inactivation were observed; P1 occurred initially and was followed by P2, characterised by a slower rate of inactivation. Together, P1 and P2 accounted for the inactivation of the large proportion (approximately 5 log CFU.mL<sup>-1</sup>) of the initial population (approximately 8.0 log CFU.mL<sup>-1</sup>). A third, more rapid phase of inactivation (P3) then became evident and continued until viable cells could no longer be detected.

Biphasic inactivation of bacterial populations in response to inimical non-thermal constraints has been widely reported (Gustafson *et al.*, 1998; Shadbolt *et al.*, 1999; Casey and Condon, 2002; Shadbolt, 2004). In those instances, the first phase of inactivation occurs at a more rapid rate than the second and, thus, these phases are analogous to P1 and P2 reported in the current work. The investigations of Shadbolt *et al.* (1999) and Shadbolt (2004) are of particular relevance to the present work because they too describe the inactivation of *E. coli* M23 in response to low  $a_w$  environments. Those reports demonstrated an initial, rapid decline that accounted for the inactivation of around 90% of the starting population within approximately five hours. That was followed by a slower phase of inactivation, described as tailing. During the current investigation, an initial, rapid phase of inactivation was also observed (P1) but the rate of decline was less, and the duration of this phase longer, than that described by Shadbolt *et al.* (1999) and Shadbolt (2004). The differences in the characteristics of this phase of inactivation



are not attributable to the use of different suspension broths (TSB in this study or NB in Shadbolt *et al.*, 1999) because previous investigations by the candidate using NB showed no analogous phase of decline (Section 2.3.1.2; Figure 2.4). Instead, P1 and P2 described in this work appear to correspond to the initial phase of inactivation and the tail population, respectively, described by Shadbolt *et al.* (1999) and Shadbolt (2004) albeit with somewhat different characteristics. The existence of an initial, rapid phase of inactivation that is followed by a slower rate of decline is probably due to inherent variability in the population (Moats *et al.*, 1971; Cerf, 1977). That is, the abrupt and detrimental change in environmental  $a_w$  initially eliminates many cells rapidly (during P1), whereas, some cells are either more resistant, or are able to adapt to the stressful environment and are not inactivated until P2.

The final, more rapid phase of inactivation (P3) demonstrated in this work appears similar to behaviour described by Brown (2002) in *E. coli* M23 exposed to pH 3.5. The existence of this third phase of inactivation is likely to be hidden if the inactivation of bacterial populations is not followed to the minimum level of detection or where that minimum detection level is relatively high. It should be noted, however, that the confidence in data corresponding to  $< 2.0 \log \text{CFU.mL}^{-1}$  (and therefore a large proportion of P3) is limited unless experiments are replicated (Cerf, 1977), as in this study. While deviations from log-linear inactivation are considered to be a real phenomenon (Humpheson *et al.*, 1998), even slight variations in methods used can alter observed deviations and this may also contribute to the general lack of P3-like inactivation phases reported in the literature.

The existence of P3 during low  $a_w$ -induced inactivation of *E. coli* might result from exhaustion of reserves or accumulation of toxins in the environment. The former could be tested by replenishing energy resources (e.g. by adding simple sugars to the broth) during P2 and determining its influence on subsequent inactivation. To determine if toxin build-up causes P3, the population could be resuspended in fresh medium during P2 and the inactivation kinetics examined for changes. Otherwise, enhanced inactivation of *E. coli* during P3 might be due to a self-mediated process of cell death that has been described to occur in *E. coli* in response to other, non-thermal stresses (Hazan *et al.*, 2004). This is examined in Chapter 5 of this thesis.

From a practical perspective, the identification of the specific conditions that cause an increase in the rate of bacterial inactivation in surviving subpopulations (in this case characterised by P3) in response to a constant inimical condition (in this case  $a_w$  0.90) could be of benefit to the food industry, particularly during the manufacture of products that are held at non-lethal temperatures for long periods (e.g. some UCFM products, hard cheese). Incorporation of such conditions, or their further exploitation, within food processing practices has the potential to maximise the reduction in the initial bacteria load and, thereby, reduce microbial foodborne illness.

### **3.4.2 Evaluation of the Inactivation of *Escherichia coli* in Response to Low Water Activity and in Combination with Low pH**

In most foods with extended shelf lives, several factors contribute to the production of a microbiologically safe product. The combination of  $a_w$  and pH reduces the risk of foodborne illness associated with UCFM and thus it is important to investigate the collective effect of these lethal agents on *E. coli*. Shadbolt *et al.* (2001) reported

that the lethal effect of both low  $a_w$  and low pH on the inactivation of *E. coli* M23 was not additive, thus challenging the widely-held view that the combination of these parameters are important in bacterial inactivation in UCFM manufacture (Leistner and Rödel, 1976; Leistner, 1994). Survival curves were generated in the present study to confirm the findings of Shadbolt *et al.* (2001). The inactivation kinetics of populations of *E. coli* M23 exposed to  $a_w$  0.90 (NaCl as the humectant) in combination with pH 3.5 (HCl as the acidulant) closely resembled that of pH 3.5 alone, supporting the conclusion of Shadbolt *et al.* (2001) that, for the specific  $a_w$  and pH levels used, the low  $a_w$  constraint was not an important determinant of *E. coli* survival.

These findings are in direct contrast to those of Entani *et al.* (1998) that suggested that the lethal effect of low  $a_w$  or low pH (acetic acid as the acidulant) is enhanced when the two stresses are applied simultaneously. In that study, the additional antimicrobial activity of the organic acid under acidic conditions could have influenced the response. Conversely, Casey and Condon (2002) demonstrated that the presence of NaCl actually protects *E. coli* against a simultaneously applied acid challenge by increasing the internal pH of the bacterial cell. In the latter case, the use of considerably lower NaCl concentrations and organic acids as acidulant may have caused the different response to that observed in the present work and that of Shadbolt *et al.* (2001).

Importantly, results presented in Chapter 2 of this thesis show that the effect of low  $a_w$  and low pH on the inactivation of *E. coli* M23 is highly dependent on the suspending medium employed for the stress treatment (i.e. the extracellular environment during stress application). While simultaneous exposure to low  $a_w$  and low pH in TSB did not enhance inactivation of *E. coli* M23 in comparison to the pH

stress alone, inactivation was increased in three other media tested (LB, NB and MM-B). These results suggest that, short of determining the protective effect of TSB, this particular aspect of combined stress treatments would be better determined using the food matrix itself.

In food manufacturing processes, the exposure of foodborne bacteria to different stresses does not necessarily occur simultaneously. For example, in UCFM manufacture, NaCl is added initially and thus the  $a_w$  is abruptly lowered. The pH of UCFM, however, gradually decreases due to the production of organic acids during fermentation and the  $a_w$  is reduced further during the drying stage of manufacture. Depending on the exact process, the  $a_w$  may not reach growth-limiting levels until this late stage (Leistner, 1994). Because of the variability in UCFM processes, it is important to understand the influence of the order of exposure to stresses on pathogens potentially present so as to recognise more hazardous processes and to optimise their safety.

In a study of the significance of the order of stress application, Shadbolt *et al.* (2001) reported that exposure of *E. coli* M23 to low pH in TSB followed by a low  $a_w$  treatment more rapidly reduced viable numbers than when the order of stresses was reversed. During the present study it was noted that the addition of NaCl to low pH broth caused the pH of the medium to be further reduced. In TSB, the pH dropped from 3.5 to 3.1 following the addition of 6 – 8 g NaCl (required to reduce the  $a_w$  of the solution to 0.90; data not shown). Although no such reduction was reported in Shadbolt *et al.* (2001), the observation presented here suggests that the 'low  $a_w$ ' treatment applied as a SLS in that work actually involved low  $a_w$  in combination with a more severe acid challenge. The candidate postulated that the increased severity of the acid stress might have caused the enhanced rate of

inactivation reported when acid stress preceded osmotic challenge. Accordingly, this hypothesis was tested. Two *E. coli* M23 populations were exposed to pH 3.5 for 24 hours and NaCl was then added as a SLS. Experiments used MM-B so that, because of its reduced buffering capacity, a larger drop in pH (from 3.5 to 2.9) resulted following addition of NaCl. While both populations displayed a rapid rate of decline immediately following imposition of the SLS, no resistant subpopulation appeared when the pH of the broth was reduced to 2.9 by addition of NaCl. In contrast, when the drop in pH was readjusted to 3.5, a resistant subpopulation was evident at approximately 28 hours (at a population density of approximately 5 log CFU.mL<sup>-1</sup>) and these cells were subsequently inactivated at a rate similar to the control that used combined  $a_w$  and pH as a FLS.

These results indicate that the imposition of a more severe acid stress (pH 2.9) during application of an osmotic SLS ( $a_w$  0.90) enhances the inactivation of *E. coli* M23 compared to the analogous treatment with pH 3.5. This highlights the importance of clearly defining the stresses imposed on bacterial populations. Having addressed the impact of the additional acid stress on the inactivation of *E. coli* M23, it is apparent from the current work that exposure of *E. coli* M23 to an acid stress prior to treatment with  $a_w$  0.90 (with the pH of the broth readjusted to 3.5) is still more lethal than when the order of stresses is reversed. Thus, this work supports the results of Shadbolt *et al.* (2001).

The findings of Shadbolt *et al.* (2001) further suggest that the inactivation of *E. coli* M23 is enhanced by the imposition of a first and second lethal stress rather than the simultaneous application of the same stresses. Those authors suggest that exposure to an initial stress sensitises the cells to a subsequent stress, possibly by placing an energy burden on the cell (Peleg and Cole, 1998; Shadbolt *et al.*, 2001).

In contrast, the results of the present study using MM-B rather than TSB, indicate that simultaneous exposure to  $a_w$  0.90 and pH 3.5 inactivates *E. coli* M23 at a similar rate as exposure to pH 3.5 (as a FLS) and then  $a_w$  0.90 (with pH readjusted to 3.5; as a SLS) after imposition of the SLS. Therefore, at least in MM-B, acid stress does not appear to sensitise the cells to subsequent stress. Further, in this work *E. coli* M23 initially treated with an osmotic stress followed by an acid shock as a SLS were, interestingly, not inactivated as rapidly as the combined low  $a_w$  and low pH control. This suggests that initial exposure to low  $a_w$  actually protects a proportion of the population against subsequent acid shock, possibly by selecting for physiological variants better able to survive stress. Future experiments could test this hypothesis by varying the time at which the SLS (pH 3.5) is applied to *E. coli* populations treated with  $a_w$  0.90 as a FLS and analysing the inactivation kinetics. If prior exposure to low  $a_w$  selects for physiological variants that are better able to survive the subsequent acid challenge, then shorter exposure to  $a_w$  0.90 will decrease the proportion of those variants in the population when the SLS is introduced and enhance subsequent inactivation.

The current results further show that the inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 and pH 3.5, whether those stresses be imposed simultaneously or separately, contain a stage in which the rate of inactivation of the population is relatively slow. These stages occur when the population density is between 3.5 and 5.5 log CFU.mL<sup>-1</sup> and suggest that although exposure to multiple stresses causes an immediate rapid decline in the bacterial population, a proportion of the population is better able to survive the combined stress. The size of this subpopulation and the exact rate and duration of this slower phase of inactivation are dependent on the order of the applied stresses or whether they were imposed simultaneously. Because the rate of inactivation of the population increases again at some point,

this enhanced ability to survive is apparently limited and the subsequent increase in inactivation rate might be due to a build-up of cellular damage with exposure time or a lack of available resources required for continued survival. By replenishing energy resources during the slower phase of inactivation and examining the effect of this on the population's survival, the latter could be tested in a manner similar to that detailed in Section 3.4.1 to examine the cause of P3.

### **3.4.3 Examination of Injury in *Escherichia coli* in Response to Osmotic and/or Acid Stress**

Inactivation kinetics in populations of *E. coli* M23 exposed to osmotic and acid stress has been presented above. From these results, pH 3.5 appears to be more lethal in *E. coli* M23 than treatment with  $a_w$  0.90, based on the treatment time required to reduce the population to the minimum level of detection using culture-based enumeration. Subsequent investigations studied the level of injury (as measured by sensitivity to bile salts) within the viable proportion of *E. coli* M23 exposed to these treatments and revealed that damage induced by either low  $a_w$  or low pH is not necessarily lethal. Secondly, the injury profile of acid-stressed *E. coli* M23 was very different to that of the low  $a_w$ -treated cells and suggests that the mechanisms involved in the action of these stresses on *E. coli*, or responses to them, are distinct. Cells that survived the acid challenge displayed less damage than their osmotically stressed counterparts and that damage was less dependent on exposure time. This increased level of injury in low  $a_w$ -treated cells would, presumably, place more stress on surviving cells because barriers to the extracellular environment are compromised, toxin-degrading enzymes are disrupted and/or efflux systems are non-functional. Therefore, the low  $a_w$ -treatment appears to place a greater burden on cells that survive the initial challenge, although this is limited in context to

cellular components that can be measured via bile salt sensitivity and does not consider injury to other cellular sites. Further, while the current investigation suggests that osmotic stress affects the cell envelope, enzymes and efflux systems more so than low pH, this is not necessarily the case because the methods used cannot assess *lethal* damage to those components. Therefore, either stress may cause inactivation by damaging those systems but this was beyond the scope of the current work.

#### **3.4.4 Conclusions**

Results presented in this chapter show that although the inactivation kinetics of *E. coli* M23 in response to  $a_w$  0.90 differs somewhat between replicate experiments conducted at different times, survival curves show three phases of inactivation. The first phase is characterised by a relatively fast rate and accounts for the inactivation of the majority of the population, whereas the second phase involves a slower rate of decline. The final, rapid phase of inactivation occurs when the population density is low and so might be overlooked in some studies if viability is not assessed until the minimum level of detection is reached or when that level is high. The ability to induce this latter phase of inactivation during food manufacturing processes would improve the microbiological safety of foods by further reducing the number of pathogens present. Depending on the laboratory medium employed, the inactivation of *E. coli* M23 in response to combined  $a_w$  and pH stresses may or may not be enhanced from those individual treatments. Leistner and Rödel (1976) suggested that the combination of these inimical conditions improves the level of inactivation of *E. coli* in UCFM products and the order in which osmotic or acid challenges are applied to *E. coli* has been reported to have a strong influence on the subsequent reduction in viable numbers (Shadbolt *et al.*, 2001). The current work



confirmed that application of a low pH stress followed by an osmotic challenge inactivates *E. coli* M23 more rapidly than the reverse order of stress application. It was apparent that *E. coli* M23 cells initially treated with an osmotic stress were better able to survive a subsequent acid shock. Such findings may be of benefit in the design of food manufacturing processes. Finally, the level of injury in non-thermally stressed *E. coli* M23 populations was shown to be greater in osmotically stressed cells than their acid challenged counterparts, which highlights a difference in the mode of action of these agents.

This study contributes to the current understanding of the kinetics of inactivation of *E. coli* in response to low  $a_w$  environments and is of relevance to food manufacturing processes that rely on non-thermal inactivation, such as UCFM, because experiments considered the effects of stresses both singly and in combination. The applicability of these findings to the production of microbiologically safe UCFM is developed further in the following chapter.

## **Chapter 4**

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# **Modelling Inactivation of *Escherichia coli* in Uncooked, Comminuted Fermented Meat using Broth-Based Systems**

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## 4.1 INTRODUCTION

### 4.1.1 Uncooked, Comminuted Fermented Meat

UCFM, or simply fermented meat, encompass a large group of food products in which raw, ground meat is preserved by the process of fermentation and drying. Having been manufactured for centuries as a means of preserving an otherwise highly perishable commodity (Lücke, 1985; Ricke *et al.*, 2001), UCFM are now popular throughout the world. The *per capita* production and consumption is greatest in Europe (Lücke, 1985), where products are categorised as either dry (salami) or semi-dry (cervelat). In the US, pepperoni is the most popular UCFM variety, consumed in sandwiches and in large volumes as pizza topping (Hinkens *et al.*, 1996), whereas the Asian UCFM market is dominated by pork-based products including the moist Thai-style fermented sausage, *nam* (Petchsing and Woodburn, 1990; Yu and Chou, 1997). In Australia, numerous and varied UCFM varieties are available based largely on European and Asian UCFM styles and methods.

### 4.1.2 Manufacture of Uncooked, Comminuted Fermented Meat

UCFM products tend to be 'ready-to-eat' foods made by mixing meat and fat into a batter also containing salt, carbohydrates, curing agents, spices and a starter culture and stuffing into casings. The pH of the product is lowered during fermentation and the  $a_w$  reduced initially by the addition of salt and also by drying of the product. A heat treatment is rarely included and, thus, the microbiological safety of UCFM tends to rely upon non-thermal parameters, namely low  $a_w$  and low pH. Those ingredients or processes involved in UCFM manufacture that effect the microbiological safety of these foods are discussed below with particular focus on the fate of *E. coli*.

#### **4.1.2.1 Ingredients**

UCFM production predominantly uses beef and pork, however lamb, mutton, venison and poultry meats are also in use (Ricke *et al.*, 2001). The type of meat used imparts many of the product's characteristic flavour, texture and colour. It appears to be less important from a microbiological safety perspective except where the level of pathogen contamination differs significantly between meat types. All of these meat types can be contaminated with pathogenic *E. coli*, primarily due to faecal contact during the slaughtering process (Chapman *et al.*, 1997; Keene *et al.*, 1997). A number of studies (Gill *et al.*, 1998; Heuvelink *et al.*, 1999; Sofos *et al.*, 1999; Bouvet *et al.*, 2002; Tutenel *et al.*, 2003; Albonetti *et al.*, 2004; MLA, 2005) have investigated the level of *E. coli* contamination, either on the carcass or in raw meats available for sale, and it appears that no one meat type is particularly prone to *E. coli* contamination. Studies of the level of carcass contamination have reported that beef and pork are occasionally contaminated with pathogenic *E. coli* and the level of contamination on sheep carcasses is slightly higher (Chivell, 1995; MRC, 1996; MLA, 1998; MLA, 2005). Similarly, surveys of retail raw meats and poultry have shown *E. coli* O157:H7 in 2 - 4% of ground beef, 1.5% of pork and poultry, and 2% of lamb (Doyle and Schoeni, 1987; Sekla *et al.*, 1990). In all cases, it is recommended that the meat used in UCFM manufacture should be of high quality, with low pathogenic loads.

NaCl, or common salt, is included in the batter at around 2.4 - 3.0% (weight per weight) (Lücke, 1994). The antimicrobial activity of salt is related to its ability to reduce  $a_w$ , however, at this concentration the reduction in  $a_w$  is usually not great enough to be completely inhibitory to the growth of pathogens. The  $a_w$  of UCFM is further reduced, and typically to levels that inhibit the growth and survival of pathogenic bacteria, as fermentation progresses and during the drying process,

discussed later (Section 4.1.2.2). Although the amount of fat used in UCFM has no direct impact on the survival of pathogenic organisms, the meat to fat ratio has some effect on the  $a_w$  of the product. Because lean meat has a higher water content than fat, large amounts of fat in UCFM effectively reduce the amount of water in the product (Ricke *et al.*, 2001). Therefore, addition of NaCl on a weight per weight basis will result in a greater decrease in  $a_w$  if the meat to fat ratio is low.

Starter cultures consisting of a single bacterium or a cocktail of lactic acid bacteria are inoculated in the batter to high levels (e.g. 6 – 7 log CFU.g<sup>-1</sup>) so as to dominate the microbiota of the UCFM (Lücke, 1994). These bacteria metabolise carbohydrates to produce lactic acid and thereby reduce the product's pH and minimise the potential for growth of any pathogenic bacteria present. A number of bacteria are used in UCFM fermentations including *Lactobacillus sakei*, *Pediococcus acidilactici* and *Pediococcus pentosaceus*. The use of a starter culture is now compulsory in Australia to ensure a rapid pH reduction and to minimise the potential for growth of pathogens (ANZFA, 1996). Because raw meat naturally contains relatively little glucose, the addition of carbohydrates (0.4 – 0.8% weight per weight), usually as simple sugars, is essential in providing a sufficient carbon source for fermentative bacteria to reduce the pH of UCFM to low levels (Lücke, 1985).

Nitrite is usually added to UCFM at levels of 40 - 150 ppm in the sodium or potassium salt form or as nitrate, which is converted to nitrite by microbes within the starter culture (Lücke, 1985; Ricke *et al.*, 2001). The principal role of nitrite in UCFM production is the development of the pink/red colour and characteristic flavour and texture. It is important to the safety of the product primarily because it inhibits the growth and toxin formation of *Clostridium botulinum* (Davidson, 2001). The effect of nitrite on other bacteria is variable. Casey and Condon (2000) have

reported that nitrite exerts a concentration-dependent bactericidal effect on *E. coli* when present above 100 ppm in low pH broth and in UCFM. However, a study by Gibson and Roberts (1986) showed that, at concentrations typical of UCFM, nitrite caused limited inhibition of *E. coli* at various NaCl concentrations and pH values. Thus, the role of nitrite as a significant hurdle to pathogenic *E. coli* is uncertain.

#### **4.1.2.2 Processing**

The first stage in UCFM manufacture typically involves mixing high quality raw meat trimmings with the batter at low temperatures to avoid fat smearing. The mixture is blended to reduce particle size and vacuum stuffed into a semipermeable casing to minimise the presence of oxygen (Ricke *et al.*, 2001). The UCFM is then incubated at or near the starter culture's optimum temperature until a specific pH (typically 4.6 - 5.8) is reached or until carbohydrate utilisation is complete. This process is termed fermentation and is crucial from a microbiological safety viewpoint because most pathogenic bacteria are inactivated, or their growth is suppressed, by the low pH (Lücke, 1994). Adding to the antimicrobial effect of low pH, protein binding occurs during fermentation as conditions become acidic, which causes a loss in water and thereby further reduces the  $a_w$  of the product. The reduced  $a_w$  is the principal means of preventing the growth of any *E. coli* present in the UCFM (Casey and Condon, 2000).

Depending on the UCFM type, the product may be treated in various ways following fermentation. In the US, a heat treatment is sometimes applied to inactivate the starter culture and eliminate pathogens. This post-fermentation heating involves holding the internal temperature at  $> 50^{\circ}\text{C}$  for over an hour and thus the product is not strictly uncooked and is termed low temperature cooked sausage (Calicioglu *et al.*, 1997). The majority of UCFM products do not include a post-fermentation heat

treatment because of the associated changes in the product's organoleptic properties. Some UCFM are smoked heavily after fermentation. Phenols within the smoke have been shown to have antimicrobial activity however the effect of these is minimal and smoking is primarily used to enhance flavour (Ricke *et al.*, 2001).

The final step in UCFM manufacture is a drying (or maturation) process. The product is incubated at a specific temperature (usually 12 - 15°C) until the desired moisture content is achieved. This typically correlates to a  $a_w$  between 0.85 and 0.94. Although various ingredients and other processes used in the manufacture of UCFM contribute to a reduced  $a_w$ , the drying stage is crucial for achieving a  $a_w$  that is inhibitory to pathogenic bacteria.

#### **4.1.3 Foodborne Disease Associated with Uncooked, Comminuted Fermented Meat**

UCFM have long held a good safety record. Even without a heat treatment, pathogenic bacteria initially present on the meat are inhibited in UCFM, primarily because of the characteristic low pH and  $a_w$ . Foodborne disease caused by bacteria of the genera *Salmonella* and *Staphylococcus* and associated with the consumption of UCFM was largely related to slow or incomplete fermentations, a problem that has since been resolved (Bacus, 1997). More recently, however, several outbreaks of disease following consumption of UCFM and involving pathogenic *E. coli* have called into question the safety of these products.

Glass *et al.* (1992) recognised the potential for pathogenic *E. coli* to survive the processes of salami manufacture. Two years later, the first cases of foodborne disease caused by EHEC and associated with UCFM were recognised in the US (CDC,

1995a; Tilden *et al.*, 1996). These outbreaks involved 17 cases of *E. coli* O157:H7 infection in California and Washington State and included the hospitalisation of three people. The salami product implicated was produced from a single facility using a fermentation and drying program that met existing industry and regulatory standards (Tilden *et al.*, 1996).

In December 1994 and January 1995, contaminated mettwurst produced by a single manufacturer in South Australia caused a much larger outbreak of disease (CDC, 1995b). This outbreak was caused by *E. coli* O111:NM and resulted in the hospitalisation of 23 children and the death of a five year old girl. A further 210 people suffered severe effects of food poisoning. In that instance, several process failures and manufacturing 'short-cuts' were noted that probably caused the disease outbreak (Chivell, 1995). *E. coli* disease associated with UCFM have since been reported in Canada (Williams *et al.*, 2000; MacDonald *et al.*, 2004).

In an attempt to prevent further outbreaks from occurring, various national regulators instituted new guidelines for the production of UCFM. The Australia New Zealand Food Authority introduced a number of requirements including that:

"The process of fermentation and any other subsequent processes must reduce prior to sale from the processing factory by 99.9% or greater the number of *Escherichia coli* organisms potentially present in an uncooked comminuted meat product." (ANZFA, 1996)

This correlates to a 1000-fold (or 3-log) reduction in *E. coli*. The equivalent regulation recommended by the US Department of Agriculture Food Safety and Inspection Service requires a 5-log decline in *E. coli* numbers unless an approved



hazard analysis and critical control point (HACCP) plan is in place. The ability of current manufacturing processes to meet such requirements has since been investigated.

#### **4.1.4 Inactivation of *Escherichia coli* in Uncooked, Comminuted Fermented Meat**

Numerous reports have shown that standard UCFM processes are unable to achieve a 3-log reduction in *E. coli* numbers (Glass *et al.*, 1992; Clavero and Beuchat, 1996; Grau, 1996; Faith *et al.*, 1997; Calicioglu *et al.*, 2002). Hinkens *et al.* (1996) demonstrated that fermentation at 36°C to a pH  $\leq$  5.0 of pepperoni inoculated with *E. coli* O157:H7 resulted in less than 1-log reduction in pathogen numbers. A similar level of decline has been reported to occur during fermentation for various UCFM products (Calicioglu *et al.*, 1997; Faith *et al.*, 1997; Chikthimmah *et al.*, 2001; Pidcock *et al.*, 2002). Exceptions where a greater level of *E. coli* inactivation has been reported involve higher fermentation temperatures, low final pH and/or a high concentration of preservatives (Petchsing and Woodburn, 1990; Riordan *et al.*, 1998). In most cases, these processing conditions are specific to a particular UCFM type and cannot be applied more extensively because of the associated alterations to the product's flavour, colour and/or texture.

The process of maturation further causes destruction of *E. coli*. Lahti *et al.* (2001) reported that aging of a dry UCFM at 17°C for five days caused a 0.42-log reduction in *E. coli* numbers. A number of groups have reported similar levels of inactivation, usually within the range of 0.4 - 0.9 log units (Grau, 1996; Faith *et al.*, 1997; Faith *et al.*, 1998a; Faith *et al.*, 1998b). Therefore, standard fermentation and

maturation processes, even in combination, are unlikely to achieve a 3-log reduction in *E. coli*.

The capacity for new treatments or alterations to standard processing practices to further suppress *E. coli* in UCFM has been, and continues to be, investigated. Promising alternatives for the safe production of UCFM include post-fermentation heating (Ellajosyula *et al.*, 1998; Hinkens *et al.*, 1996) and extended periods of storage prior to sale (Clavero and Beuchat, 1996; Nissen and Holck, 1998; Chikthimmah and Knabel, 2001; Sharma *et al.*, 2004). However, because these treatments can cause undesirable changes to the final product, manufacturers often prefer not to implement such changes.

In response to the predominant view that most UCFM processes are unable to achieve a 3-log reduction in *E. coli* without specialised and potentially detrimental treatments, a number of national food regulators reassessed the guidelines pertaining to UCFM processes. In 2003, Food Standards Australia New Zealand amended the standard for UCFM manufacture that reflected a shift away from rigid processing guidelines including the largely unachievable requirement for a 3-log kill. Under the new standard, manufacturers are required to implement HACCP-based programs, which allow them more flexibility in meeting food safety objectives. To some extent, the design and implementation of appropriate HACCP-based plans has been difficult due to the limited understanding of the response of *E. coli* to non-thermal constraints in general and more specifically to conditions in UCFM. The work described in the present chapter attempted to build on the existing knowledge regarding the response of *E. coli* to conditions relevant to UCFM. A broth model was developed to mimic the inactivation of *E. coli* observed in UCFM and used to generate inactivation rate data. This data was analysed to determine patterns in

response and the effect of specific variables were included in a predictive model to allow for comparisons with in-product trials obtained from an independent source.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Bacterial Strains, Reagents, Media, Solutions and Equipment**

Details of bacterial strains, chemical reagents, bacteriological media, solutions and equipment (including software) are given in Appendix A. The non-pathogenic *E. coli* M23 was the test organism used for all experiments described in this chapter.

### **4.2.2 General Methods**

#### ***4.2.2.1 Preparation of Stationary Phase Populations of Escherichia coli***

Five colonies of *E. coli* M23 on TSA (A.1.1) were inoculated to 10 mL TSB in a McCartney bottle and incubated statically at 37°C for 24 hours. The cell suspension was diluted 1/1000 in 50 mL TSB in a 250 mL Erlenmeyer flask and incubated at 25°C in a water bath with shaking at 60 oscillations per minute for 24 hours, to achieve a viable count of approximately 9.0 CFU.mL<sup>-1</sup>.

#### ***4.2.2.2 Preparation of Broth for the Simulation of Uncooked, Comminuted Fermented Meat Conditions***

As appropriate, to a stock solution of specified medium prepared according to the method described in Appendix A.1.3, lactic acid was added to a final concentration of 150 mM. The  $a_w$  was reduced to the required level by the addition of NaCl. The pH of the solution was subsequently adjusted by the addition of 10 M HCl or 10 M NaOH. The solution was divided into two aliquots. To one volume, the availability of oxygen was reduced by the addition of sodium thioglycolate to a final

concentration of 0.1%. Volumes (18 mL) were transferred to test tubes containing a 5 mm magnetic stirrer bar and plugged with cotton wool and autoclaved (121°C, 20 minutes). Before the solution could cool, approximately 5 mL molten sterile 'vaspar' (a 1:1 mixture of petroleum jelly and paraffin oil) was overlayed onto the solution to inhibit oxygen diffusion. These broths provided a 'reduced oxygen' environment (as assessed by the methodology described later in Section 4.2.3.2). 'Standard oxygen' broths were prepared from the remaining aliquot of stock solution by transferring 18 mL volumes to L-tubes, which were plugged with cotton wool and autoclaved (121°C, 20 minutes). All tubes were incubated at the appropriate temperature for several hours to equilibrate to temperature prior to inoculation.

#### **4.2.2.3 Inoculation of *Escherichia coli* to Broth**

A 2 mL aliquot of a stationary phase population (4.2.2.1) of *E. coli* M23 was transferred to an 18 mL volume of broth (4.3.2.2), providing a 1/10 dilution of the original stationary phase inoculum. The population was uniformly distributed throughout the broth by the magnetic stirrer (for reduced oxygen broths) or by gentle agitation (for standard oxygen broths). Broths were incubated at the appropriate temperature either statically in a water bath (for reduced oxygen broths) or in a temperature-gradient incubator with shaking at 60 oscillations per minute (for standard oxygen broths). Immediately following inoculation, a 2 mL aliquot was withdrawn for  $a_w$  and pH measurements.

#### **4.2.2.4 Enumeration of Viable Cells, Construction of Survivor Curves and Determination of Inactivation Rates**

Immediately prior to the inoculation of *E. coli* M23 to broth, and at regular intervals afterwards, the viability of each population was estimated by culture-based enumeration as described in Section 2.2.2.4. That method was modified slightly

when sampling from reduced oxygen broths, in that a magnetic stirrer was used to mix the solution prior to removal of the aliquot and any break made in the vaspar layer was resealed by gentle heating the vaspar layer from above with a Bunsen flame. Inactivation curves were constructed by plotting  $\log_{10}\text{CFU.mL}^{-1}$  against time. Assuming log-linear inactivation kinetics, the rate of inactivation of *E. coli* and the y-intercept were determined by linear regression analysis using Microsoft® Excel.

### **4.2.3 Broth Model Development and Characterisation**

#### **4.2.3.1 Comparison of Cooked Meat Medium- and Brain Heart Infusion-Based Broth Models**

Stationary phase populations (4.2.2.1) of *E. coli* M23 were inoculated to cooked meat medium (CMM)- or brain heart infusion (BHI)-based broths (4.2.2.3) with varying temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability conditions. The number of viable cells was estimated periodically by culture-based enumeration and the rate of inactivation for each treatment was determined (4.2.2.4). These inactivation rates were compared to those predicted by the predictive model named PRMS01 described in Ross and Shadbolt (2001) by plotting the natural logarithm of the rates of inactivation of *E. coli* as a function of the reciprocal of absolute temperature in an Arrhenius plot.

#### **4.2.3.2 Monitoring Variables in the Broth Model**

A stationary phase population (4.2.2.1) of *E. coli* M23 was inoculated to CMM-based broth (4.2.2.3) [ $a_w$  0.91, pH 4.5, 150 mM lactic acid, 20°C, standard oxygen conditions]. The  $a_w$  and pH of the broth was monitored periodically for 20 days. In a separate experiment, reduced oxygen broths [ $a_w$  0.91, pH 4.5, 150 mM lactic acid] were prepared using MM as described in Section 4.2.2.2 except that resazurin, a

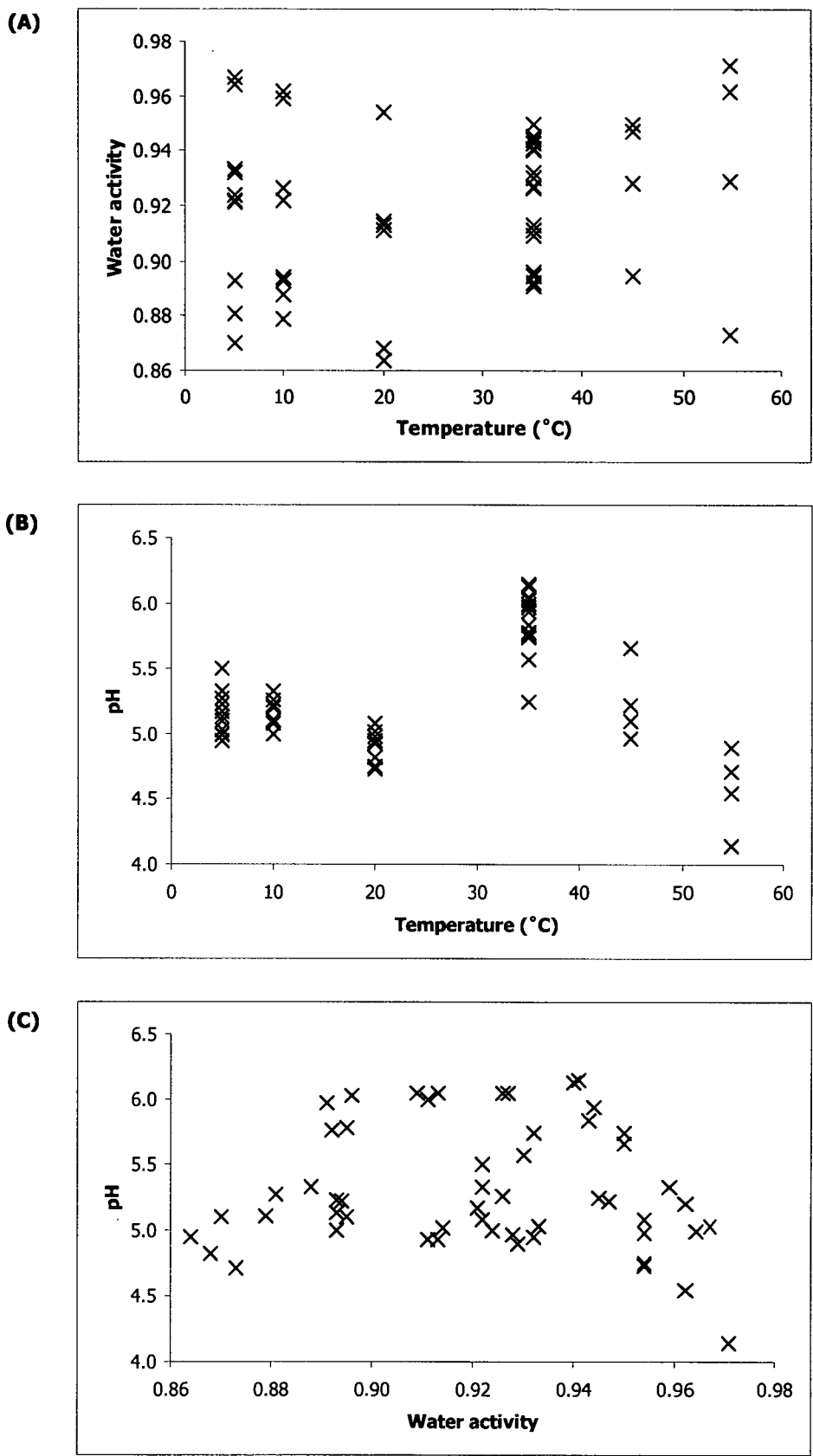
colourless compound that becomes pink when oxidised, was added to a final concentration of 0.5%. The broths were inoculated with stationary phase populations (4.2.2.1) of *E. coli* M23 and incubated statically at 5, 20 or 45°C (4.2.2.3). The vaspar layer was pierced with a pipette tip daily to simulate the removal of a sample for enumeration of *E. coli*. Gentle heating from above the vaspar layer with a Bunsen flame immediately resealed any break in that layer. The broth was monitored daily for a colour change from colourless to pink, indicative of an increase in the presence of oxygen within the broth and therefore loss of the reduced oxygen environment.

**4.2.4 Generation of Inactivation Rates of *Escherichia coli* using the Broth Model**

Stationary phase populations (4.2.2.1) of *E. coli* M23 were inoculated to CMM-based broth (4.2.2.3) with various combinations of incubation temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability. The number of viable cells was determined by culture-based enumeration and the rate of inactivation and the y-intercept were calculated from each survival curve (4.2.2.4). A set of 62 inactivation rate data was generated for the ranges of conditions shown in Table 4.1 and the combinations of temperature,  $a_w$  and pH values depicted in Figure 4.1.

**Table 4.1** Ranges of conditions for which the inactivation rate of *E. coli* M23 was determined in the broth model. A total of 62 inactivation rates were determined.

| Variable                  | Range for which the inactivation rate of <i>E. coli</i> was determined |
|---------------------------|--|
| Temperature               | 5, 10, 20, 35, 45 or 55°C  |
| Water activity            | 0.864 – 0.971  |
| pH                        | 4.14 – 6.15  |
| Lactic acid concentration | 0 or 150 mM  |
| Oxygen availability       | Standard or Reduced  |



**Figure 4.1** Combinations of (A)  $a_w$  and temperature, (B) pH and temperature and (C) pH and  $a_w$  for which inactivation rates of *E. coli* M23 were determined using the broth model.

#### 4.2.5 Determination of the Number of Phases in *Escherichia coli* Inactivation

Studies by Brown (2002) have indicated that the low pH-induced inactivation of *E. coli* M23 can involve a rapid decline in viability that commences when the population density is around  $6 \log \text{CFU.mL}^{-1}$ . A similar, rapid phase of *E. coli* M23 inactivation was induced by low  $a_w$ , as described in Chapter 3 of the current work. To determine if environmental conditions relevant to UCFM cause a similar pattern in inactivation, the survival curves generated by the methodology described in Section 4.2.4 were visually analysed to determine the number of phases of inactivation of *E. coli* M23. Where multiple phases of inactivation were evident, the inactivation rate of each phase was calculated (4.2.2.4).

#### 4.2.6 Effect of Variables on the Inactivation Rates of *Escherichia coli*

The inactivation rates of *E. coli* M23 determined by the methodology described in Sections 4.2.4 (based on linear regression analysis of the entire curve) and 4.2.5 (that used multiple rates of inactivation) were analysed for the effect of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability. The analysis was not improved by the use of multiple inactivation rates and so that data (from Section 4.2.5) was omitted. The inactivation rates as a function of temperature were plotted as Arrhenius plots and analysed by linear regression to the data for temperatures  $\leq 45^\circ\text{C}$  using Microsoft® Excel. The residuals of the fitted line were calculated by subtracting the  $k$ (inactivation rate) observed in the broth model from that 'predicted' by the fitted equation (i.e. the 'temperature-only' model). Those residuals were plotted against the  $a_w$ , pH, lactic acid concentration or oxygen availability value of the treatment. Linear regression analysis was carried out using Microsoft® Excel to identify any relationship between the residuals and the tested



variable. For any strong relationship, an appropriate term was included in the fitted model and the residuals for those models were determined and analysed as described above. For lactic acid concentration and oxygen availability effects, significance ( $P \leq 0.01$ ) was tested by single factor analysis of variance using Microsoft® Excel. A synergistic effect of temperature and  $a_w$  was tested by plotting  $\ln(\text{inactivation rate})$  observed at temperatures  $\leq 45^\circ\text{C}$  against the  $a_w$  of the treatment. Lines of best fit to each data set were determined using Microsoft® Excel. The  $\ln(\text{inactivation rate})$  predicted for a single  $a_w$  value (0.918) was calculated from the fitted equations and plotted against temperature.

#### **4.2.7 Inclusion of Water Activity and pH Terms in the Predictive Model PRMS05. $a_w$ /pH**

Using the set of 58 inactivation rates determined using the methodology described in Section 4.2.4 for incubation temperatures  $\leq 45^\circ\text{C}$ , a new predictive model was generated by Dr. T. Ross (Pers. comm., 2005) using UltraFit software. This model, called PRMS05. $a_w$ /pH, included temperature,  $a_w$  and pH as predictor variables.

##### **4.2.7.1 Evaluation of the Predictive Model PRMS05. $a_w$ /pH**

The effect of including  $a_w$  and pH as predictor variables in the predictive model PRMS05. $a_w$ /pH was determined by comparing this model with the temperature-only model generated by the methodology described in Section 4.2.6. These models were evaluated against inactivation rate data generated using the broth model at temperatures  $\leq 45^\circ\text{C}$  using the methodology described in Section 4.2.4 or in UCFM. The UCFM data set (survival curves) were provided by Dr. P. Vanderlinde (Pers. comm., 2003) and the candidate determined the rate of inactivation for each survival curve by linear regression analysis using Microsoft® Excel. Specifically, the

$\ln$ (inactivation rate) predicted by each model was plotted against the  $\ln$ (inactivation rate) data observed in the broth system or in UCFM. The percent variance in the data was determined using Microsoft® Excel. The mean square error (MSE) in the data compared to the model was determined by squaring the difference in the  $\ln$ (inactivation rate) data observed with those predicted by each model and calculating the average.

## 4.3 RESULTS

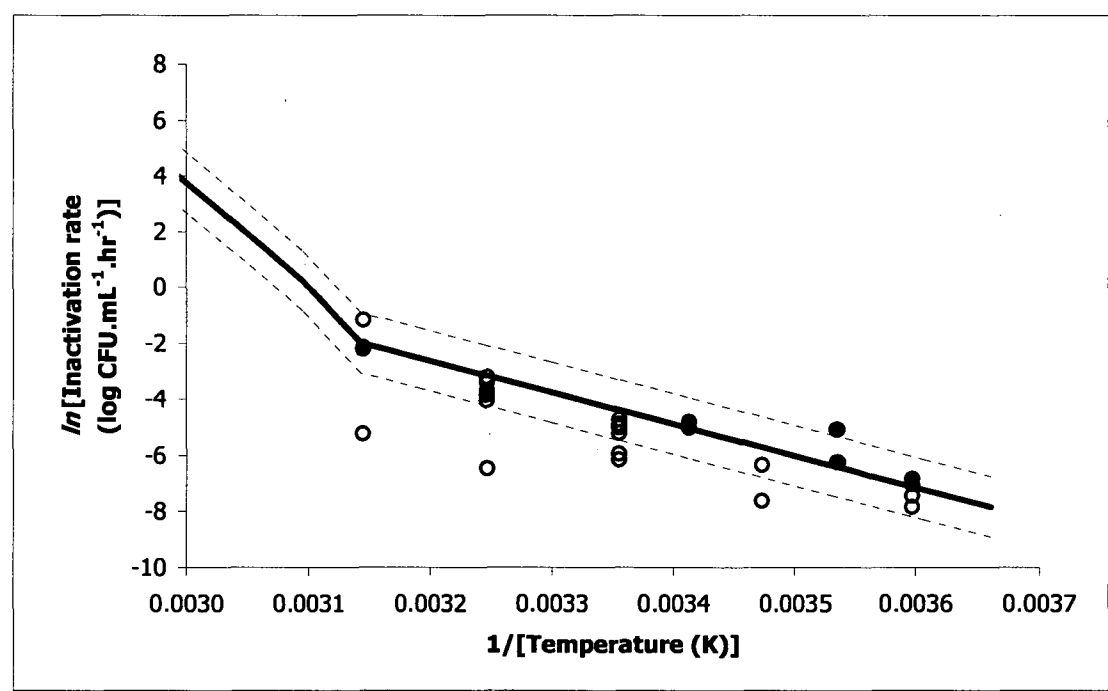
### 4.3.1 Broth Model Development and Characterisation

#### 4.3.1.1 Comparison of Cooked Meat Medium- and Brain Heart Infusion-Based Broth Models

Two separate broth models were initially developed to simulate the inactivation of *E. coli* in UCFM that used different media (CMM without pellets and BHI). Using these model systems, the inactivation of *E. coli* M23 in response to a limited number of combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability was investigated. The survival curves are given in Appendix B.1.1. Assuming log-linear inactivation kinetics, the lines of best fit to the data were used to calculate the inactivation rate. A set of 10 inactivation rate data for *E. coli* M23 in the CMM-based model and a set of 22 inactivation rate data for *E. coli* M23 in the BHI-based model were determined and are presented in Appendix B.1.2.

To determine which of the two broth models better described the inactivation of *E. coli* expected in UCFM, the inactivation rate data sets were compared to a predictive model (PRMS01) previously developed (Ross and Shadbolt, 2001). PRMS01 was generated using inactivation rate data obtained from a variety of published and unpublished sources that reported on the inactivation of *E. coli* in UCFM. Thus, a

comparison of the inactivation rates observed using the two broth models developed in the current work with that model provided a means of assessing the UCFM-simulating power of the broth models. The natural logarithm of the inactivation rates predicted by PRMS01 is presented as a function of temperature in Figure 4.2. The inactivation rate data generated using the CMM- or BHI-based models is similarly shown.



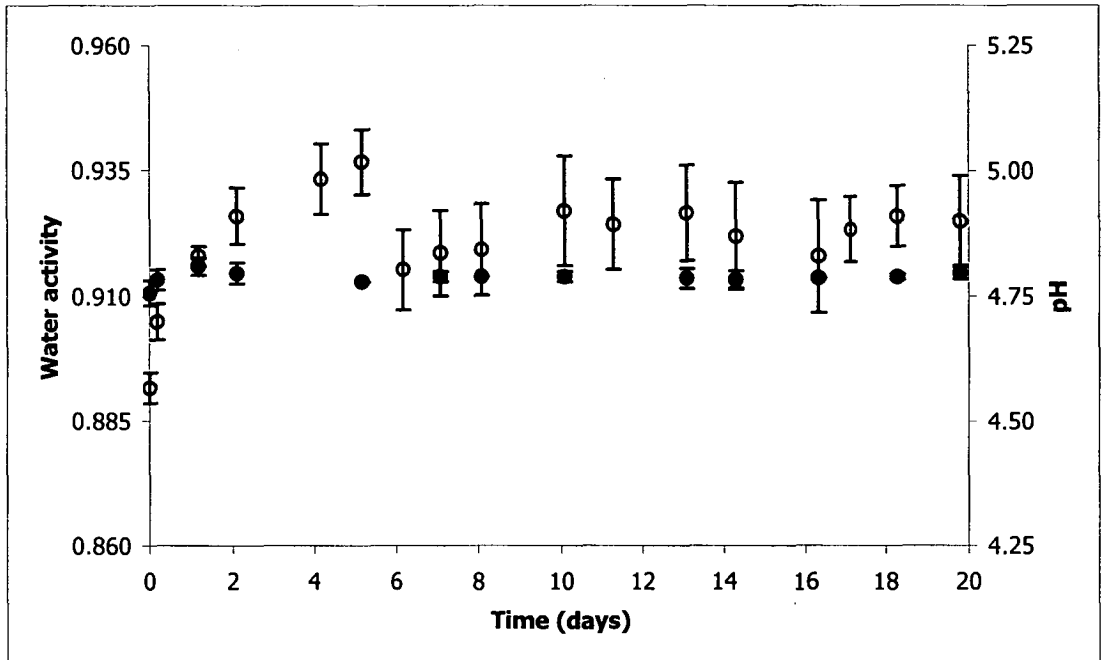
**Figure 4.2** Comparison of the inactivation rates of *E. coli* M23 observed in the CMM- (●) and BHI- (○) based broth models with that predicted to occur in UCFM. The data is shown as an Arrhenius plot [ $\ln(\text{inactivation rate})$  vs.  $1/(\text{absolute temperature})$ ]. The solid line shows  $\ln(\text{inactivation rate})$  'predicted' by the predictive model PRMS01 and the dashed lines indicate the upper and lower confidence intervals (95%) of these predictions.

Five inactivation rates generated using the BHI-based broth model fell outside the confidence intervals of the predictive model, which is equivalent to 23% of the data generated using this broth model. In contrast, the CMM-based broth model gave

only one inactivation rate datum that was not within the confidence intervals of the predictive model, corresponding to 10% of the CMM-based data set. From this, it was adjudged that the CMM-based broth model is more likely to simulate the inactivation rate of *E. coli* in UCFM than the BHI-based model. Thus, the CMM-based method, which used CMM with the insoluble pellets removed, was used as the experimental model in subsequent experiments. For simplicity, this system is hereafter referred to as 'the broth model'.

#### **4.3.1.2 Monitoring Variables in the Broth Model**

The broth model generated was anticipated to be used to determine the inactivation rate of *E. coli* in response to combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability, where these variables were defined as that measured immediately following commencement of the experiment ( $a_w$  and pH) or as set during preparation of the broth (lactic acid concentration, oxygen availability and temperature). The temperature variable was, by nature, constant throughout the course of the study. However, there was potential for the other variables to alter during the trial and so some of these factors were monitored. The pH and  $a_w$  of the broth model inoculated with *E. coli* M23 was determined at regular time intervals for 20 days (i.e. the time that the inactivation of *E. coli* was expected to be monitored at 20°C) in three separate experiments. The average values are shown in Figure 4.3.



**Figure 4.3** Fluctuations in  $a_w$  (●) and pH (○) of the broth model. The broth [ $a_w$  0.91, pH 4.5, 150 mM lactic acid, standard oxygen conditions] was inoculated with *E. coli* M23 and incubated with shaking at 20°C. The average  $a_w$  and pH determined during three separate experiments and the standard deviation ( $n = 3$ ) is shown.

Considering the measurement error of the  $a_w$  meter used ( $\pm 0.003$ ) the  $a_w$  of the broth did not alter significantly over the time monitored. In contrast, the pH of the broth system increased from the value initially set (pH 4.56) to pH 5.18 within 5.2 days. After this time the pH fluctuated between 4.83 and 4.98 until the end of the experiment. Throughout the experiment, the standard deviation of the pH was large, indicating that the increase in pH was not uniform between the three separate experiments and that those fluctuations in pH could be difficult to predict accurately. Because monitoring changes in lactic acid levels in broth can be difficult, this was not tested in the current work. The observed fluctuations in the broth pH may, however, reflect some variance in lactic acid concentration due to cellular metabolism.

The broth model could be prepared to provide a standard or reduced oxygen environment. The latter was achieved by (a) the addition of sodium thioglycolate, an oxygen scavenger, to the broth, (b) incubating the tubes statically and (c) pouring vaspar over the broth to inhibit the diffusion of oxygen. To assess whether these factors could maintain a reduced oxygen environment for the time over which the inactivation kinetics of *E. coli* were determined, resazurin, a colourless compound that becomes pink when oxidised, was added to the medium. The time taken for the solution to turn pink, considered to be the time to loss of reduced oxygen conditions, was measured.

**Table 4.2** Time to loss of reduced oxygen conditions in the MM-based broth model [ $a_w$  0.91, pH 4.5, 150 mM lactic acid, reduced oxygen conditions] incubated at 5, 20 or 45°C in three separate experiments.

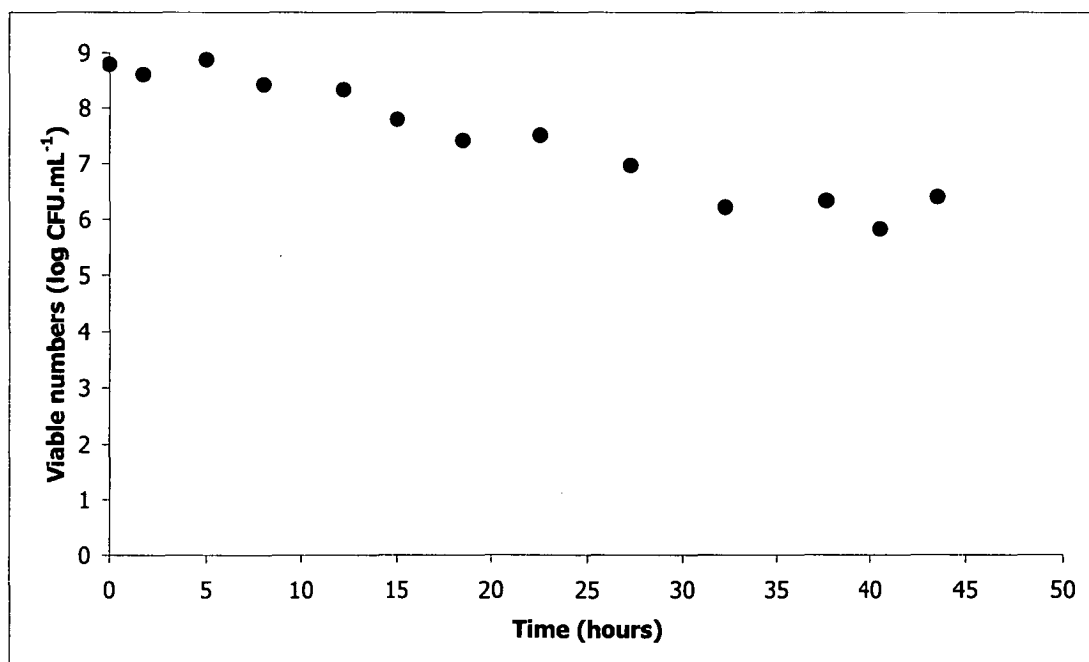
| Temperature<br>(°C) | Time to loss of reduced oxygen conditions (days) |                |             |                           |
|---------------------|--|----------------|-------------|---------------------------|
|                     | <i>Minimum</i>                                   | <i>Maximum</i> | <i>Mean</i> | <i>Standard deviation</i> |
| 5                   | 42   | 61             | 53          | 9.7                       |
| 20                  | 49   | 73             | 62          | 12                        |
| 45                  | 45   | 55             | 50          | 5.0                       |
| All temperatures    | 42   | 73             | 55          | 9.7                       |

These results indicate that the methodology used could achieve a reduced oxygen environment but that this was lost after some time. The temperature of incubation did not appear to effect the time to loss of reduced oxygen conditions. Although on average, reduced oxygen conditions were maintained for 55 days, in one case this was lost within 42 days. Therefore, adopting a 'worse case' view, the reduced oxygen broth model was considered unreliable from 42 days following inoculation with *E. coli*. Consequently, the number of viable cells was not determined following

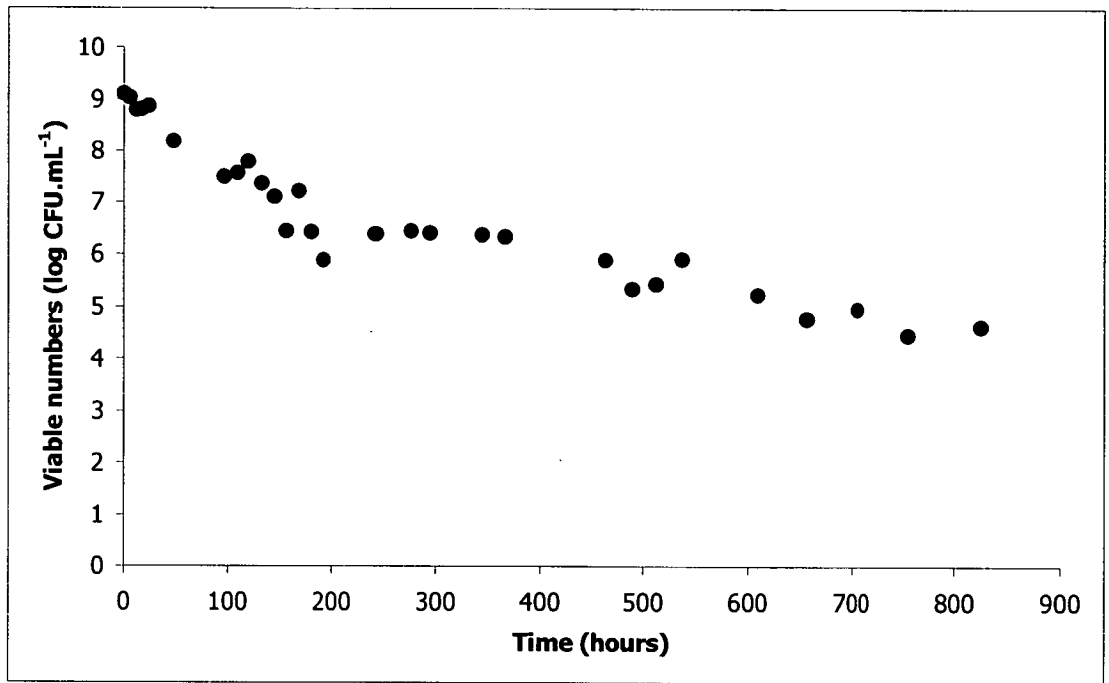
this time period (i.e. after approximately 1000 hours) for subsequent inactivation studies.

#### 4.3.2 Determination of the Number of Phases of Inactivation of *Escherichia coli* in Response to Uncooked, Comminuted Fermented Meat-Simulated Conditions

Using the broth model, the inactivation kinetics of *E. coli* M23 in response to UCFM-simulated conditions were determined. The inactivation kinetics for all combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability tested are shown in Appendix B.2.1. Analysis of these results illustrate that the inactivation of *E. coli* M23 occurred at a constant rate or followed biphasic inactivation kinetics. An example of each of these inactivation patterns is shown in Figures 4.4 and 4.5. A third phase of inactivation was not evident under any of the combinations of conditions tested in the broth model.



**Figure 4.4** Single phase inactivation kinetics observed in a population of *E. coli* M23 exposed to  $a_w$  0.895, pH 5.78 and without lactic acid in the broth model at 35°C under reduced oxygen conditions. The population declines at a constant rate over the entire duration of the trial.



**Figure 4.5** Biphasic inactivation kinetics observed in a population of *E. coli* M23 exposed to  $a_w$  0.879, pH 5.11 and 150 mM lactic acid in CMM at 10°C under standard oxygen conditions. The population declines at an initial, rapid rate followed by a slower rate of inactivation (i.e. after approximately 180 hours).

### 4.3.3 Effect of Variables on the Inactivation Rates of *Escherichia coli*

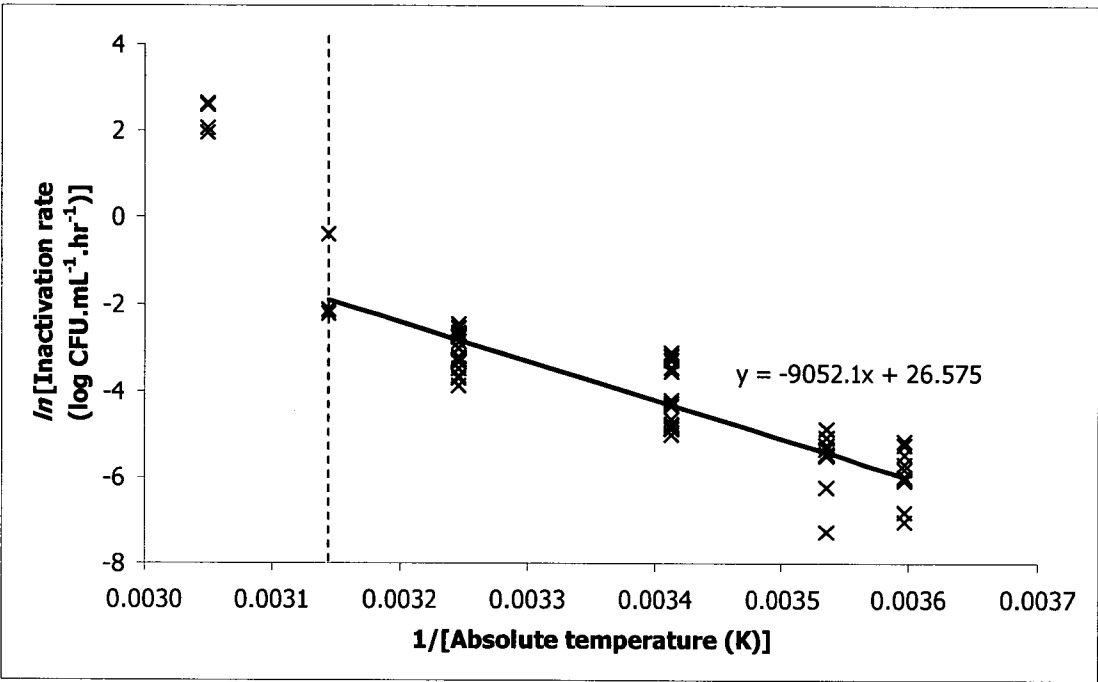
The rates of inactivation of *E. coli* M23 were calculated from the inactivation kinetics given in Appendix B.2.1, assuming log-linear inactivation. A set of 62 inactivation rate data was generated and is summarised in Appendix B.2.2. The individual effects of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability on the rate of inactivation of *E. coli* M23 were analysed and are discussed below.

#### 4.3.3.1 Temperature

Ross and Shadbolt (2001) identified the usefulness of plotting the natural logarithm of inactivation rates as a function of the reciprocal of absolute temperature (i.e. an



Arrhenius plot). Thus, the calculated rates of inactivation of *E. coli* M23 are presented as an Arrhenius plot in Figure 4.6 for all combinations of variables tested.



**Figure 4.6** The effect of temperature on the rate of inactivation of *E. coli* M23 in the broth model at various combinations of  $a_w$ , pH, lactic acid concentration and oxygen availability. The data is shown as an Arrhenius plot [ $\ln(\text{inactivation rate})$  vs.  $1/(\text{absolute temperature})$ ]. The dashed line corresponds to 45°C. The solid line is a linear regression fitted to the data for temperatures  $\leq 45^\circ\text{C}$  ( $r^2 = 0.8104$ ) and the equation is shown.

From the Arrhenius plot shown in Figure 4.6, it is clear that temperature has a strong effect on the rate of inactivation of *E. coli* M23. A simple linear regression fitted to the data for temperatures  $\leq 45^\circ\text{C}$  gave a correlation co-efficient ( $r^2$ ) for the fitted equation of 0.8104, indicating that the effect of temperature explains 81.0% of the variance in the observed  $\ln(\text{inactivation rate})$  data. At each temperature, however, a large amount of variation in the data remains and the level of variance appears to be independent of the temperature value.

To investigate the source of the remaining variability in the  $\ln$ (inactivation rate) data the residuals of the fitted line (i.e. a temperature-only model) were calculated. The residual is the difference between the response predicted by the fitted equation and the response actually observed. A positive residual indicates that the temperature-only model over-predicts the response, while a negative residual indicates that the model under-predicts the observed response. The magnitude of the residual indicates the extent of over- or under-prediction. A relationship between the residuals and any of the remaining independent variables indicates that that variable has a strong influence on the observed response.

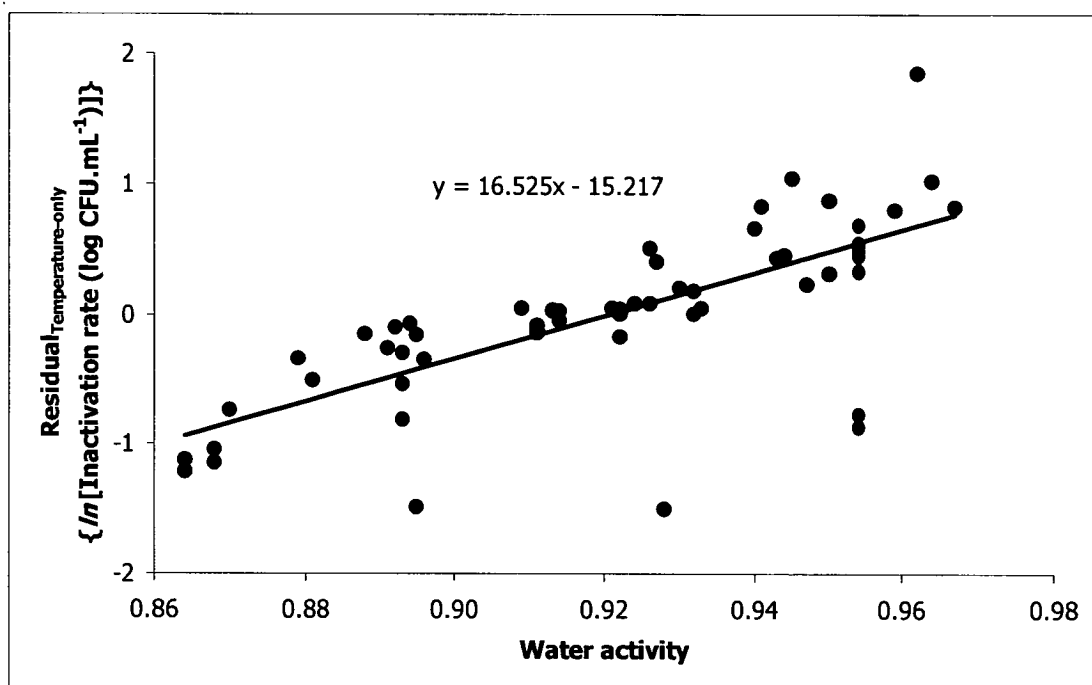
#### **4.3.3.2 Water Activity**

The residuals were plotted against  $a_w$  (and thereby were normalised for temperature) to reveal correlations as shown in Figure 4.7. This analysis indicates that  $a_w$  strongly correlates with over- or under-prediction of the temperature-only model ( $r^2 = 0.5254$ ). Thus,  $a_w$  is an important factor affecting the rate of inactivation of *E. coli* M23 within the range of variables tested in this study using the broth model and, consequently, temperature and  $a_w$  terms were combined in the following fitted model:

$$\ln(\text{inactivation rate}) = -9052.1 \times (1/T) - 16.525 \times a_w + 41.792$$

where, temperature (T) is measured in Kelvin and  $a_w$  has its usual meaning.

This temperature and  $a_w$  model accounted for 91.1% of the variance in the broth model inactivation rate data (data not shown).



**Figure 4.7** Residuals of the temperature-only model vs.  $a_w$  of treatments. The line of best fit to the data and equation is shown ( $r^2 = 0.5254$ ).

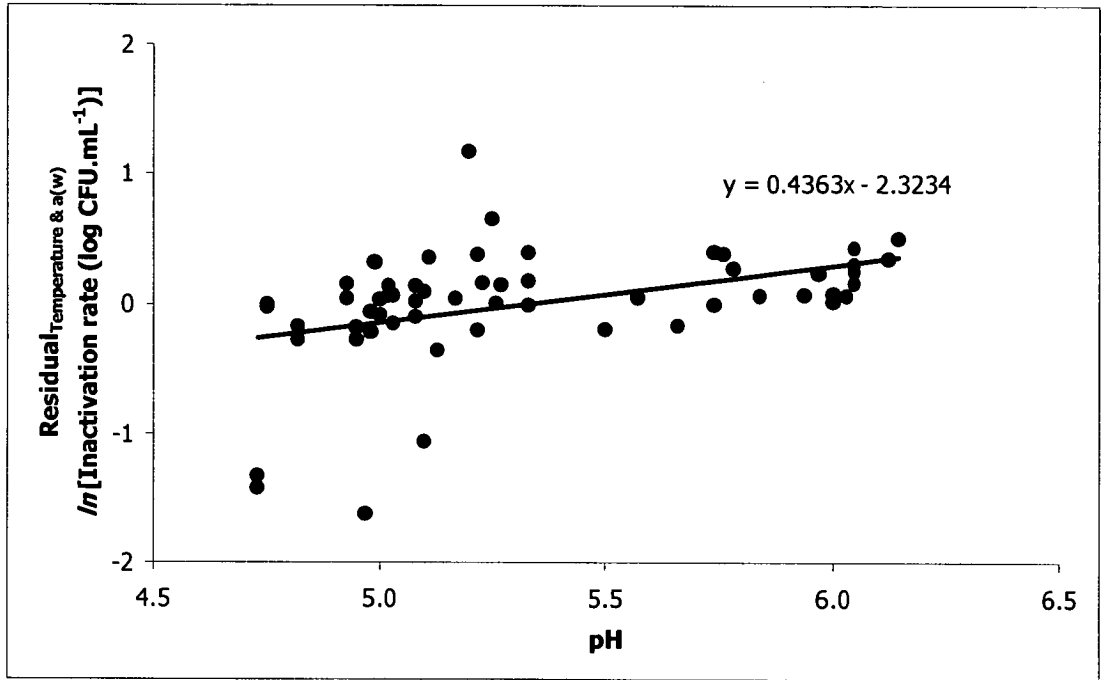
#### 4.3.3.3 pH

When the residuals of the temperature and  $a_w$  fitted model were plotted against pH, as shown in Figure 4.8, only a slight correlation between pH and the residuals of the temperature and  $a_w$  model ( $r^2 = 0.1770$ ) was observed. Incorporation of the pH term into the temperature and  $a_w$  model is shown below:

$$\ln(\text{inactivation rate}) = -9052.1 \times (1/T) - 16.525 \times a_w - 0.4363 \times \text{pH} + 44.1154$$

where, temperature (T) is measured in Kelvin and  $a_w$  and pH have their usual meaning.

The incorporation of the pH term in the above fitted model improved the variance accounted for in the broth model data from 91.1 to 93.7% (data not shown).



**Figure 4.8** Residuals of the temperature and  $a_w$  model vs. pH of treatments. The line of best fit to the data and equation is shown ( $r^2 = 0.1770$ ).

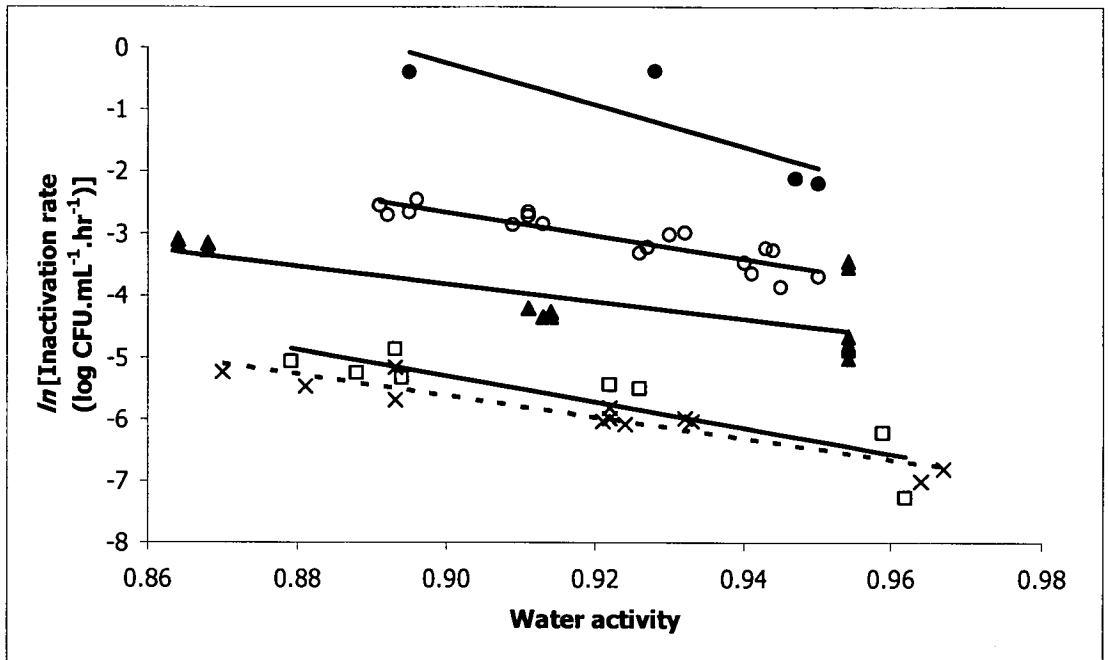
#### 4.3.3.4 Other Variables

The effect of lactic acid concentration (0 or 150 mM) and oxygen availability (standard or reduced) on the inactivation of *E. coli* M23 were analysed similarly to  $a_w$  and pH except that significance was tested by a single factor analysis of variance using Microsoft® Excel. By these analyses (data not shown) neither of these variables was found to have a significant affect on the rate of inactivation of *E. coli* M23 for the range of factors considered.

#### 4.3.3.5 Synergistic Effect of Temperature and Water Activity

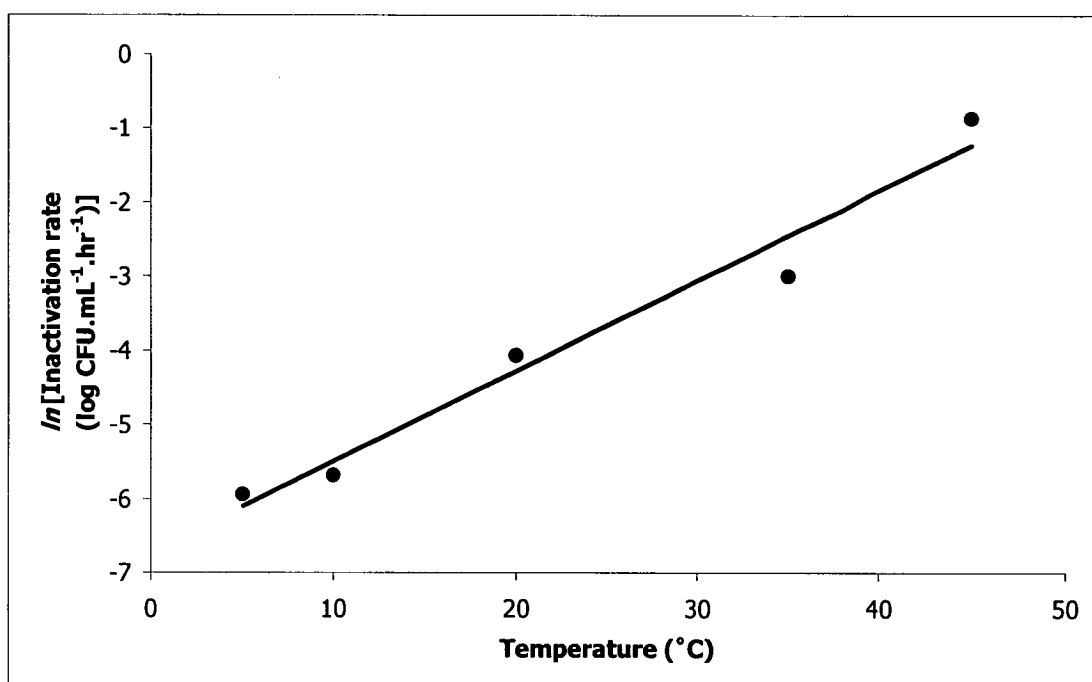
Given that both temperature and  $a_w$  were observed to have a strong effect on the inactivation of *E. coli* M23 in UCFM-simulated conditions using the broth model, the relationship between these variables was tested. Figure 4.9 shows the relationship

between  $a_w$  and  $\ln(\text{inactivation rate})$  for temperatures  $\leq 45^\circ\text{C}$  tested in the broth system.



**Figure 4.9** The relationship between  $a_w$  and  $\ln(\text{rate of inactivation of } E. coli)$  in the broth model for various temperatures ( $\bullet$   $45^\circ\text{C}$ ,  $\circ$   $35^\circ\text{C}$ ,  $\blacktriangle$   $20^\circ\text{C}$ ,  $\square$   $10^\circ\text{C}$  and  $\times$   $5^\circ\text{C}$ ). The lines of best fit to the data are shown (— 45, 35, 20 and  $10^\circ\text{C}$ ; or ---  $5^\circ\text{C}$ ).

Lower  $a_w$  increases the  $\ln(\text{inactivation rate})$  at all temperatures in an almost identical manner as shown by the fitted lines (simple linear regression), given in Figure 4.9, for all five data sets. From the fitted lines for the 5, 10, 20, 35 and  $45^\circ\text{C}$  data sets, the  $\ln(\text{inactivation rates})$  predicted for  $a_w$  0.918 (the median of  $a_w$  levels tested in the broth model) were calculated and are shown in Figure 4.10. The relationship between temperature and  $\ln(\text{inactivation rate})$  is well described by a straight line ( $r^2 = 0.969$ ) indicating that there is no synergy between the effects of temperature and  $a_w$  on the  $\ln(\text{inactivation rate})$  term.



**Figure 4.10** Predicted  $\ln(\text{inactivation rate})$  derived from the fitted lines in Figure 4.9 (based on  $a_w$ ) vs. temperature at  $a_w$  0.918. The line of best fit to the data is shown ( $r^2 = 0.969$ ).

#### 4.3.4 Inclusion of Water Activity and pH Terms in the Predictive Model

##### PRMS05. $a_w$ /pH

Using the set of 58 inactivation rates generated from the broth model (omitting those that were incubated at 55°C), a new predictive model (called PRMS05. $a_w$ /pH) was generated by Dr. T. Ross (Pers. comm., 2005). This model included temperature,  $a_w$  and pH predictor variables. The fitted model is shown below:

$$\ln(\text{inactivation rate}) = -10582.6 \times (1/T) - 17.3481 \times a_w - 0.73594 \times \text{pH} + 51.66895$$

where, temperature (T) is measured in Kelvin and  $a_w$  and pH have their usual meaning.

#### 4.3.4.1 Evaluation of the Predictive Model PRMS05.a<sub>w</sub>/pH

The effect of including the a<sub>w</sub> and pH terms in PRMS05.a<sub>w</sub>/pH was assessed by comparison of this model with its corresponding temperature-only model detailed in Section 4.3.5.1 and described as:

$$\ln(\text{inactivation rate}) = -9052.1 \times (1/T) + 26.575$$

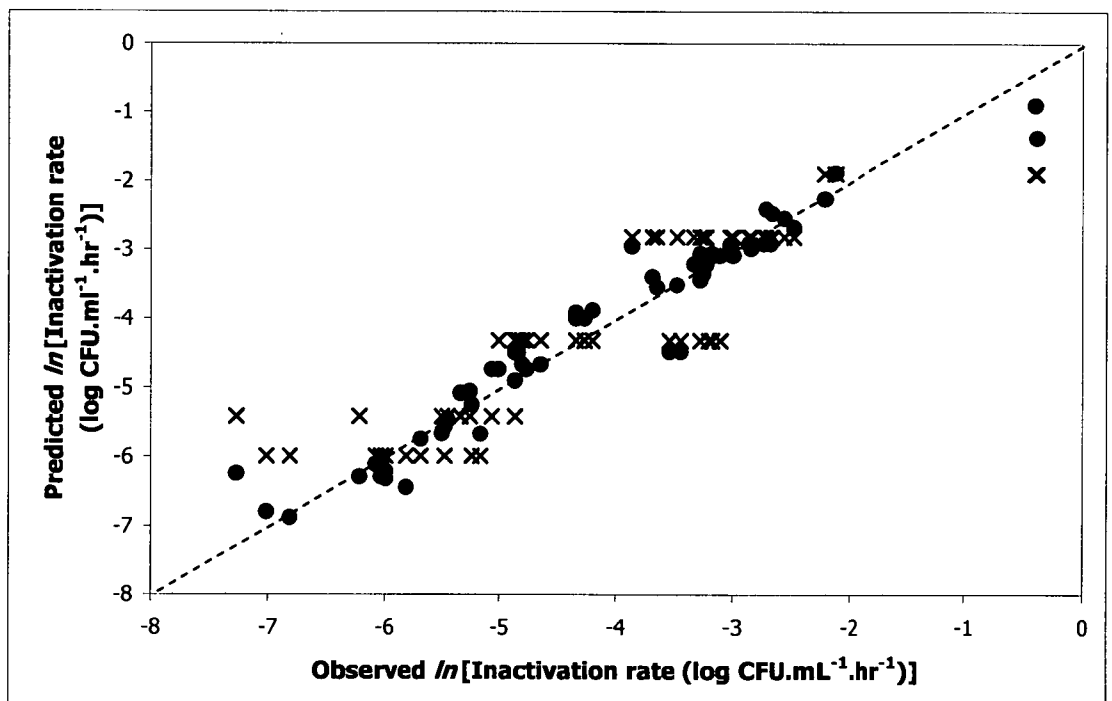
where, temperature (T) is measure in Kelvin.

The performance of these models was evaluated using observed inactivation rate data sets obtained from the broth system (i.e. the set of 58 inactivation rates used to generate the fitted models) and in UCFM. The latter data set used survival curves that were provided by Dr. P. Vanderlinde (Pers. comm., 2003). The full UCFM data set is summarised in Appendix C. The goodness of fit of the observed inactivation rate data generated using the broth system or in UCFM to each fitted model (PRMS05.a<sub>w</sub>/pH and the temperature-only model) was assessed by calculating the MSE. A perfect description of the data will give an MSE of zero. The variance in the observed data accounted for by each fitted model was also determined. The results of these analyses are shown in Table 4.3.

**Table 4.3** Evaluation of the performance of the PRMS05.a<sub>w</sub>/pH and temperature-only models against inactivation rate data observed in the broth system and in UCFM.

|                                | <b>Broth system</b>                 |            | <b>UCFM</b>                         |            |
|--------------------------------|-------------------------------------|------------|-------------------------------------|------------|
|                                | <b>% variance<br/>accounted for</b> | <b>MSE</b> | <b>% variance<br/>accounted for</b> | <b>MSE</b> |
| <b>PRMS05.a<sub>w</sub>/pH</b> | 94.2                                | 0.132      | 53.4                                | 3.08       |
| <b>Temperature-only model</b>  | 81.0                                | 0.428      | 72.4                                | 1.47       |

Evaluation of PRMS05. $a_w$ /pH using the broth system data indicates that incorporation of the  $a_w$  and pH terms to the fitted model improved the percent variance accounted for and the goodness of fit of the broth system data. The latter is represented graphically in Figure 4.11, where if the predictive models described the data exactly, the points would fall along the diagonal line shown. The incorporation of the  $a_w$  and pH predictor variables can be clearly seen to move the data closer to the diagonal line which indicates improvement in the goodness of fit of the data using PRMS05. $a_w$ /pH.

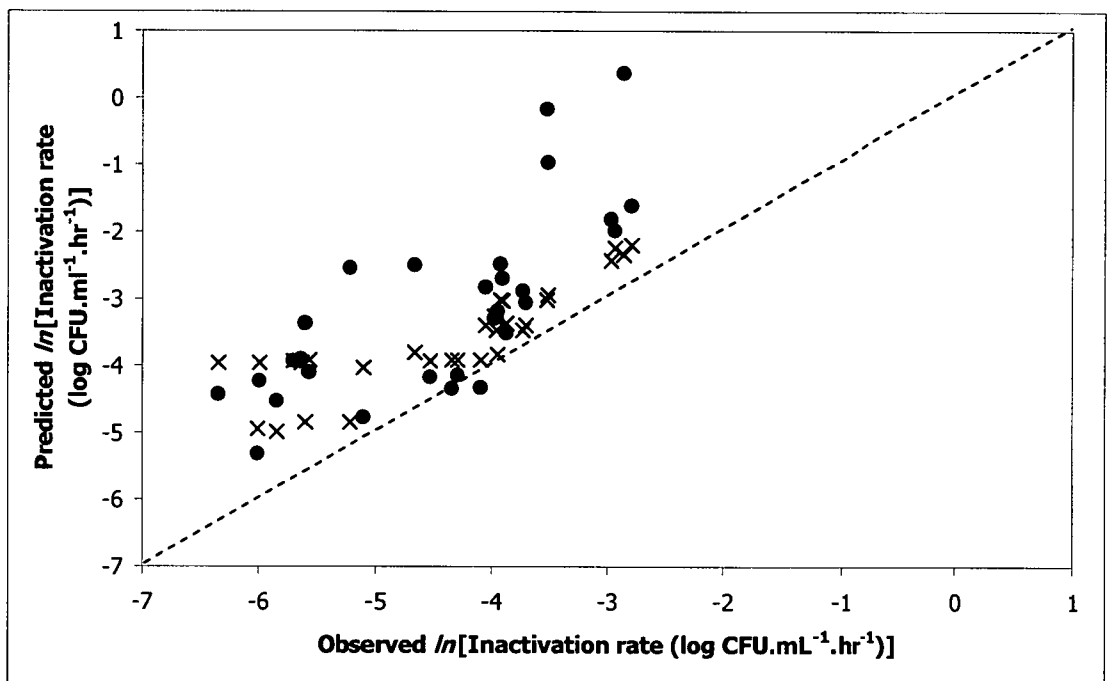


**Figure 4.11** The goodness of fit of the broth model data using the temperature,  $a_w$  and pH model, PRMS05. $a_w$ /pH (●) and the temperature-only model (x). The dashed line indicates the line of equivalence (where observed and predicted values are in perfect agreement).

The results presented in Table 4.3 further illustrate, however, that the inclusion of the  $a_w$  and pH variables in the predictive model reduced the performance of the



predictive model when evaluated against the UCFM data, as described by both the MSE and the percent variance in the data accounted for. Figure 4.12 shows the goodness of fit of the UCFM data to both of the predictive models and clearly illustrates that the temperature-only model is a better predictor of *E. coli* inactivation in UCFM, although both predictive models tend to overpredict the level of inactivation.



**Figure 4.12** The goodness of fit of the UCFM data using the temperature,  $a_w$  and pH model, PRMS05. $a_w$ /pH (●) and the temperature-only model (×). The dashed line indicates the line of equivalence (where observed and predicted values are in perfect agreement).

## 4.4 DISCUSSION

Investigating the response of *E. coli* to various environmental factors within UCFM presents numerous problems because of uncontrolled factors within the system, such as the effects of background microflora and the spatial dispersal of the test

organism. Because these studies typically involve 'destructive' sampling (i.e. a whole sausage is used to measure the number of surviving *E. coli* at a single time point), there are difficulties in maintaining accuracy during enumeration and the preparation of the UCFM is extremely labour intensive (P. Vanderlinde, Pers. comm., 2002). While such studies may be useful in evaluating the response of *E. coli* to a specific process, such investigations offer little in defining the relative contributions of processing conditions (e.g. temperatures, fermentation time, final  $a_w$  and pH, starter cultures, etc.) to the safety of the product. Therefore, they are not able to predict the safety of a specific UCFM product or process on the basis of these readily measurable properties. Consequently, a detailed understanding of the ecology of *E. coli* in response to UCFM processing is difficult to ascertain from in-product trials.

To this end, a simplified experimental (i.e. laboratory-based) model can help to reveal and quantify basic patterns of response because the number of uncontrolled variables is reduced. Furthermore, because variables can be controlled and manipulated, the data generated from a single broth model has the potential to be applicable to multiple UCFM manufacturing regimes. Also, such systems do not usually involve destructive sampling and so larger volumes of data can be obtained more readily. This is particularly useful in the evaluation of bacterial inactivation kinetics because microbial survival can be measured frequently. Once relationships are described in the model system, the more variable and complex patterns of response that occur in foods may be more readily understood.

It has been questioned whether broth models have the capacity to simulate UCFM processes (Grau, 1996). Some model systems (e.g. that described by Tomicka *et al.*, 1997) do not adequately reflect conditions and processes in UCFM manufacture

and therefore would appear unlikely to simulate the response of *E. coli* to UCFM conditions. Further, within the literature, there is a distinct lack of direct comparisons (i.e. studies that use identical methodology and similar variables) between model systems and UCFM trials, which make the assessment of model systems difficult.

One broth model that can be directly evaluated against its analogous UCFM product is that described in Casey and Condon (2000). Using a TSB-based broth model that was modified by the inclusion of salt, sucrose, ascorbate, a *P. acidilactici* starter culture and various concentrations of nitrite, those researchers determined the inactivation of *E. coli* O157 at 37°C over 48 hours. The reduction in *E. coli* O157 numbers in an analogous salami preparation fermented at 37°C for 48 hours was determined by the same methodology. A comparison of the pH levels (reliant on the production of acid by the starter culture) and the inactivation of *E. coli* O157 observed using the broth model and the laboratory-scale fermented sausage is summarised in Table 4.4.

**Table 4.4** Data of Casey and Condon (2000) showing the pH and amount of inactivation of *E. coli* O157 observed in the broth model of UCFM and an analogous fermented sausage incubated at 37°C for 48 hours.

|                                    | Initial<br>pH | Final<br>pH      | Inactivation of <i>E. coli</i> O157<br>(log CFU.unit <sup>-1</sup> ) |     |     |     |     |
|------------------------------------|---------------|------------------|--|-----|-----|-----|-----|
| <i>Nitrite concentration (ppm)</i> |               |                  | 0  | 50  | 100 | 200 | 300 |
| <b>Broth model</b>                 | 5.8           | 4.5 <sup>a</sup> | -0.3   | 0   | 1.0 | 3.0 | 3.2 |
| <b>Fermented sausage</b>           | 5.8           | 4.7              | 0.8  | 1.4 | 1.5 | 2.0 | 2.9 |

<sup>a</sup> Measured at 24 hours. It is assumed that no further reduction occurred by 48 hours.

<sup>a</sup> Measured at 24 hours. It is assumed that no further reduction occurred by 48 hours.

The results of Casey and Condon (2000) indicate that the broth model developed was useful in determining the inactivation of *E. coli* O157 during fermentation at nitrite concentrations  $\geq 100$  ppm but was unreliable when nitrite levels were lower than this. At nitrite concentrations of 200 and 300 ppm, the broth model slightly overestimated the amount of *E. coli* O157 inactivation in UCFM. Similarly, the simple model systems of Brown (2002) and Shadbolt *et al.* (1999) produced greater inactivation rates than are observed in UCFM products at equivalent temperatures and  $a_w$  or pH.

Ross and Shadbolt (2001) analysed inactivation data generated from broth systems and UCFM trials sourced from published and unpublished studies. They concluded that some broth systems do reliably mimic both qualitative and quantitative features of *E. coli* inactivation in UCFM. They stated that the maturation process in UCFM manufacture is the most likely process to be accurately represented in a model system because it is characterised by a relatively stable environment, except for a continual reduction in  $a_w$ . In contrast, the process of fermentation would appear more difficult to model, at least in a simple and 'easy-to-use' system, because various parameters, such as the concentration of sugars and organic acids, pH and  $a_w$  tend to alter quite dramatically during this stage of manufacture.

The application of results based on a model system is best reserved for when the findings generated using that model have been validated within the appropriate food matrix. Even when a model system does not entirely simulate conditions or responses observed in UCFM, useful conclusions may be made provided that the limitations of the model are recognised. With this in mind, the current work aimed to generate a model system that could mimic the inactivation of *E. coli* in UCFM, most likely during the maturation process, and to use this system to identify any

patterns in the inactivation of *E. coli* in response to an UCFM-simulated environment.

#### **4.4.1 Broth Model Development and Characterisation**

Two model systems that used different media (CMM or BHI) were initially developed. A comparison of *E. coli* M23 inactivation rates generated using these two broth models with that observed in UCFM indicated that the CMM-based broth model was better able to simulate the rate of inactivation of *E. coli* in these foods. Due to limited reliable data from UCFM trials reported in the literature, it was not possible to validate fully that this model accurately mimicked *E. coli* inactivation in UCFM. However, a comparison with the predictive model PRMS01 that described UCFM-derived data indicated that both the CMM- and BHI-based models generated *E. coli* inactivation rates at least in the correct range. By this analysis, the CMM-based broth performed better and so was used for the generation of a larger data set of *E. coli* inactivation rates. Because the analysis using PRMS01 effectively allowed the broth model data to be compared to a range of UCFM types, it is likely that the model system developed will be applicable to numerous UCFM products, rather than a specific product type or processing regime. With that said, the potential for certain effects of specific products or processes must also be considered and investigated.

The broth model developed allowed five variables (temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability) to be adjusted. Of these variables, pH was difficult to control. The pH of the broth deviated from its set value, particularly due to sterilisation by autoclaving, but also during the evaluation of *E. coli* inactivation. During a preliminary study to simulate *E. coli* inactivation in UCFM using a broth

system, Shadbolt (2004) reported a similar problem when using CMM. That author suggested that the insoluble pellets present in CMM had some effect on the absorption of hydrogen ions and for that reason the CMM pellets were removed from the broth in the current work by the method described in Appendix A.1.3. This dramatically reduced pH variations due to autoclaving (data not shown) but did not prevent fluctuations in pH during the inactivation trial. Thus, the use of the broth model required an element of operator expertise and familiarity to simulate pH conditions observed in UCFM. Although pH was not monitored throughout the majority of trials, it is recognised that fluctuations were likely to have occurred (as shown in Figure 4.3) and, for simplicity, the treatments were characterised by a single pH value (i.e. that measured immediately following inoculation with *E. coli*).

#### **4.4.2 Generation of Inactivation Rates of *Escherichia coli* using the Broth Model**

Using the broth model described, a data set of 62 rates of inactivation of *E. coli* M23 was obtained that covered a combination of variables relevant to UCFM. The ranges of variables tested (reproduced from Table 4.1) and those relevant to Australian-produced UCFM are shown in Table 4.5 for comparative purposes. The lactic acid concentrations described is similar for UCFM produced in the US and in Europe. The ranges of the variables considered in this study encompass the majority of the expected range of UCFM product parameters in Australia with the exception of lactic acid. Experiments early in the study indicated no clear effect of lactic acid concentration and so this variable was not studied in detail. Most experiments included 150 mM lactic acid, however, to make the broth model system more representative of UCFM products. The model system also allowed the level of

oxygen availability to be reduced to mimic the anaerobic conditions that predominate in UCFM.

**Table 4.5** Range of variables tested in the current study and relevant to UCFM.

| Variable                  | Range in Australian-produced UCFM <sup>a</sup> | Range for which inactivation rate data was obtained using the broth model |
|---------------------------|--|---|
| Temperature               | 4 - 40°C                                       | 5 - 55°C  |
| Water activity            | 0.83 - 0.95                                    | 0.864 - 0.971   |
| pH                        | 4.4 - 6.5                                      | 4.14 - 6.15   |
| Lactic acid concentration | 110 - 450 mM <sup>b</sup>                      | 0 or 150 mM   |

<sup>a</sup> Reproduced from Ross and Shadbolt (2001), unless otherwise stated.

<sup>b</sup> Ricke and Keeton (1997).

**4.4.3 Determination of the Number of Phases of Inactivation of *Escherichia coli* in Response to Uncooked, Comminuted Fermented Meat-Simulated Conditions**

In the manufacture of many UCFM products, the inactivation of *E. coli* is approximately equal during the processes of fermentation and maturation (Ross and Shadbolt, 2001). The inactivation kinetics of *E. coli* during maturation tends to be log-linear (Grau, 1996; Hinkens *et al.*, 1996; Cosansu and Ayhan, 2000), whereas that during fermentation has not been clearly defined. Grau (1996) has reported that *E. coli* declines at a constant rate during fermentation (single phase inactivation), whereas numerous researchers (Casey and Condon, 2000; Cosansu and Ayhan, 2000; Erkkila *et al.*, 2000) have reported multi-phasic inactivation kinetics. The latter typically involve an initial phase of inactivation that occurs at a

rapid rate and that is followed by a slower rate of inactivation. The inactivation kinetics of *E. coli* in response to UCFM-like conditions observed in the current study were predominantly either uniphasic or biphasic.

In a TSB-based broth system, Brown (2002) demonstrated a third phase of inactivation of *E. coli* M23 in response to low pH and at non-lethal temperature (25°C). These conditions are relevant to some UCFM products except that the pH tested (2.0 - 3.5) was lower than that generally observed in UCFM. The third phase of inactivation occurred at a rapid rate, similar to that observed during the first phase, and continued until the number of viable cells was below the minimum level of detection. Low  $a_w$  stress causes a similar phase of inactivation in *E. coli* M23 populations (Chapter 3). While this type of triphasic inactivation kinetics has not been reported in UCFM, if they could be induced to occur they could improve the safety of UCFM by increasing the overall level of inactivation of *E. coli* (Ross and Shadbolt, 2001).

Examination of the inactivation kinetics of *E. coli* M23 in response to UCFM conditions during the current work revealed no evidence of a third phase of inactivation. For the majority of combinations tested, however, the inactivation of *E. coli* M23 was not monitored for sufficiently long enough to completely rule out the existence of a third phase of inactivation. However, for at least seven treatments the inactivation of *E. coli* M23 was monitored for sufficient time that, had a third phase of inactivation occurred, it is likely to have been detected (refer to Figures B.18, B.19, B.22, B.23, B.41 and B.48 in Appendix B.2.1 for the survival curves and details of these treatments). Evaluation of the inactivation of *E. coli* in food matrices is required to more properly assess the likelihood of inducing a third phase of inactivation of *E. coli* in UCFM.



#### **4.4.4 Effect of Variables on the Inactivation Rates of *Escherichia coli***

Temperatures used in the manufacture of UCFM rarely exceed 50°C and, therefore, unlike many food products that use high temperature processing to eliminate microbial pathogens, the inactivation of *E. coli* in UCFM is not due to temperature but to a combination of other factors. Parameters relevant to UCFM that are known to inactivate *E. coli* include low  $a_w$ , low pH, organic acids and nitrite. The lethal effect of these agents on *E. coli* has been known for some time, however, it was not until outbreaks of disease caused by *E. coli* and associated with UCFM consumption were identified that serious attempts were made to quantify the level of *E. coli* inactivation in UCFM.

A number of investigators have since reported on the inactivation of *E. coli* during the processing of specific UCFM products (Clavero and Beuchat, 1996; Calicioglu *et al.*, 1997; Faith *et al.*, 1998a; Faith *et al.*, 1998b; Cosansu and Ayhan, 2000; Calicioglu *et al.*, 2002). In some cases, the effects of changing ingredients or processing parameters were also determined (Hinkens *et al.*, 1996; Faith *et al.*, 1997; Ellajosyula *et al.*, 1998; Chikthimmah and Knabel, 2001). These investigations allowed the lethality of a particular UCFM process to be determined and the microbiological safety of that product in regards to *E. coli* to be described. As presented, it is difficult to ascertain from these studies any patterns in the response of *E. coli* to UCFM environments or any relationships between the lethal factors. This general lack of understanding of the behaviour of *E. coli* in UCFM has made regulation of the safety of UCFM manufacture problematic and the design of HACCP plans difficult (T. Ross, Pers. comm., 2002).

Ross and Shadbolt (2001) combined and reanalysed the results of such studies and thereby identified patterns in the response of *E. coli* to UCFM processes. By that

analysis, the authors showed that when  $a_w$  and/or pH are below the minimum levels required by *E. coli* for growth, the rate at which *E. coli* is inactivated is primarily determined by the temperature of the process. It was shown that by increasing the temperature of fermentation or maturation processes the level of inactivation of *E. coli* would increase and, by association, increasing the time of fermentation or maturation processes at any temperature would also increase *E. coli* inactivation. The effect of temperature on the rate of inactivation of *E. coli* has since been directly shown in UCFM (Ross *et al.*, 2004). The predictive model PRMS01 generated in Ross and Shadbolt (2001) allowed the effect of changes in temperature and/or time to be quantified.

Other factors relating to UCFM were also determined to affect the rate of inactivation of *E. coli* in UCFM, including  $a_w$  and pH, and possibly the levels of nitrite and organic acids (Ross and Shadbolt, 2001). These factors were established to be of a lesser influence than temperature but the exact effect could not be quantified from the data available within the literature at that time. The data generated using the broth model in the current work has partly alleviated this problem and as a result the effect of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability on the inactivation of *E. coli* M23 in a UCFM-simulated environment was analysed.

The current study has reiterated the significant influence of temperature on the rate of inactivation of *E. coli* when  $a_w$  and/or pH is growth limiting. Secondly,  $a_w$  was shown to have a strong effect on *E. coli* M23 inactivation rate in the broth model in that as  $a_w$  decreases the rate of inactivation of *E. coli* M23 increases. Clavero and Beuchat (1996) have shown that the lethal effect of low  $a_w$  may significantly effect the inactivation of *E. coli* O157:H7 during long-term storage of salami. Similarly,

the effect of  $a_w$  on *E. coli* O157:H7 inactivation in Lebanon bologna has been reported (Chikthimmah *et al.*, 2001). The practical implications of these findings are described later in Section 4.4.5. Other factors tested (pH, lactic acid concentration and oxygen availability) were determined to have little effect on the rate of inactivation of *E. coli* M23 in response to UCFM conditions. In-product trials using UCFM with varying pH and  $a_w$  conditions (Ross *et al.*, 2004) have supported the current finding that pH does not have a strong effect on the inactivation of *E. coli* in this food.

As previously mentioned, the rate of inactivation of *E. coli* observed in model systems is sometimes greater than that observed in UCFM (Shadbolt *et al.*, 1999; Casey and Condon, 2000; Brown, 2002). Shadbolt (2004) proposed that the aerobic conditions characteristic of the broth models could account for these discrepancies and provided evidence that the rate of inactivation of *E. coli* using an anaerobic broth system was less than that in an analogous aerobic broth system. The increased level of oxidative damage that has been reported to occur when oxygen levels are increased (Dodd *et al.*, 1997) was proposed as the molecular basis of this phenomenon (Shadbolt, 2004). The present study has indicated that the relationship between oxygen availability and the observed rate of inactivation of *E. coli* M23 at equivalent temperature and  $a_w$  was not significant. The methodology used by Shadbolt (2004) involved an increased surface area to volume ratio in the aerobic broth, which may have overstated the lethal effect of oxygen in comparison to the current study.

#### 4.4.5 Inclusion of a Water Activity Term in the Predictive Model

##### PRMS05.a<sub>w</sub>/pH

As previously mentioned, the predictive model (PRMS01) described in Ross and Shadbolt (2001) has been used to estimate the amount of inactivation of *E. coli* in UCFM processes based on time and temperature constraints. The use of PRMS01 within the food industry has been somewhat hindered because of the large confidence intervals of the predictions. For example, if a process is estimated to reduce *E. coli* by 3 log units using PRMS01, the prediction is effectively interpreted as 2 - 4 log units of inactivation. Thus, the large amount of error in the model has restricted its use in an industrial setting (T. Ross, Pers. comm., 2002).

The results of the present study indicate that both temperature and a<sub>w</sub> have a strong effect on the rate of inactivation of *E. coli* M23 in the broth model. This suggested that a predictive model relating to the inactivation of *E. coli* in UCFM should include both temperature and a<sub>w</sub> as predictor variables. Thus, using inactivation rate data generated using the broth system in the current work, a new predictive model (PRMS05.a<sub>w</sub>/pH) was generated that included temperature, a<sub>w</sub> and pH variables (T. Ross, Pers. comm., 2005). The fitted model is shown in Section 4.3.4. Evaluation of predictive model PRMS05.a<sub>w</sub>/pH against inactivation rate data obtained using the broth model suggested that temperature and a<sub>w</sub> were important determinants of the rate of inactivation of *E. coli* in UCFM. However, evaluation of the same model against UCFM data indicated that the incorporation of a<sub>w</sub> and pH as explanatory variables actually reduced the performance of the predictive model. These findings demonstrated that a<sub>w</sub> and pH do not strongly influence the inactivation of *E. coli* in UCFM.

The reason that  $a_w$  is important to *E. coli* inactivation in broth but not in UCFM is unclear. There is some uncertainty about which values best represent the  $a_w$  of UCFM because  $a_w$  declines throughout fermentation and maturation. Ross *et al.* (2004) developed a predictive model similar to PRMS05. $a_w$ /pH that was also a poor predictor of the inactivation of *E. coli* in UCFM due to the inclusion of  $a_w$  as a predictor variable. In that report, a number of different methods for describing the  $a_w$  of each UCFM trial were conducted during the analysis of the UCFM data and none were able to account for the lack of effect of  $a_w$  on *E. coli* inactivation in UCFM (Ross *et al.*, 2004). A further consideration is that the UCFM environment is heterogeneous. As a result, some bacterial cells may be exposed to  $a_w$  levels that are higher than the average  $a_w$  of the product and as such are not inactivated as expected. Indeed, Hills *et al.* (2001) suggested that “there is not necessarily any simple relationship between  $a_w$  and microbial survival in micro-heterogeneous food systems”. Those authors demonstrated that changing the food matrix microstructure while maintaining constant  $a_w$ , pH and nutrient composition causes dramatic differences in the survival of bacterial species. That work highlights that simple correlations with bulk measurements, such as  $a_w$ , can fail to provide an adequate description of the survival response of bacteria in food systems. Alternatively, the difference in  $a_w$  effect on *E. coli* inactivation between broth and UCFM noted in this work may be related to the accuracy of the viable count methods, which is quite large in UCFM trials (1 - 3 log CFU.g<sup>-1</sup>). Further investigation is required to completely resolve this issue.

#### **4.4.6 Conclusions**

The current work used a broth model to describe the inactivation of *E. coli* M23 in response to conditions relevant to UCFM. The process of developing the broth

model was described and various limitations in its use were recognised. From the large amount of inactivation rate data generated with this system, patterns in *E. coli* M23 inactivation were observed. Firstly, a rapid rate of inactivation that has been reported to occur in *E. coli* M23 populations exposed to low pH (Brown, 2002) and low  $a_w$  (Chapter 3) was not evident in the UCFM-simulated environment. Rates of inactivation of *E. coli* M23 were most strongly influenced by the temperature of incubation, although  $a_w$  also had a strong effect. The remaining variables tested (pH, lactic acid concentration and oxygen availability) had little impact on the inactivation rate in the broth system. Using the data derived from the broth model, Dr. T. Ross (Pers. comm., 2005) developed a predictive model that included  $a_w$  and pH as predictor variables and that was evaluated as part of the current work. Results indicated that this model was a good predictor of *E. coli* inactivation in the broth model but incorporation of the  $a_w$  and pH variables actually worsened its predictive power when evaluated against in-product (UCFM) trials. The basis of this inconsistency remains to be resolved; however, it does highlight the importance of evaluating any broth-based observations against trials using the appropriate food matrix. Further to the work described in this chapter, the broth-based inactivation rate data generated in this study was used to develop another predictive model (described in Ross *et al.*, 2004) that has since been distributed to UCFM manufacturers and food safety regulators in Australia. Its use is anticipated to provide considerable assistance in the development of HACCP plans to minimise the incidence of *E. coli* disease associated with UCFM.

Investigations described in Chapters 2, 3 and 4 of this thesis were performed to provide a description of the kinetics of inactivation of *E. coli* in response to inimical constraints. The quantitative information generated from such studies can be of great benefit to the food industry, as demonstrated by the development of a

predictive model (Chapter 4) and its subsequent use among UCFM manufacturers and regulators. A more mechanistic interpretation of low  $a_w$ -induced inactivation of *E. coli* would also be of considerable value. As mentioned previously, the processes involved in the low  $a_w$ -induced inactivation of bacteria remain poorly understood. The actual mechanism(s) responsible for the destruction of *E. coli* in response to low  $a_w$  conditions has not been defined and, further, it is unclear how some cells within that population are able to survive the osmotic challenge. Enhanced knowledge in this area could lead to modifications to food manufacturing regimes to overcome such responses and to improve microbiological safety. Thus, studies described in the following chapters were undertaken to explore the mechanisms of low  $a_w$ -induced inactivation of *E. coli* (Chapter 5) and to evaluate the physiology of cells that are able to survive osmotic stress for an extended period (Chapter 6).

## **Chapter 5**

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# **Evaluating Programmed Cell Death as a Mechanism of Osmotic-Induced Inactivation**

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## 5.1 INTRODUCTION

A genetically controlled process of cell death, called programmed cell death (PCD), has been identified as a mechanism of inactivation of bacterial cells in response to various stresses (Sat *et al.*, 2001; Sat *et al.*, 2003; Hazan *et al.*, 2004). The involvement of this cell death pathway in *E. coli* in response to osmotic stress remains to be determined and is the focus of the current investigation.

PCD refers to any form of cell death that is mediated by the cell itself, regardless of the initial trigger. It is a genetically controlled process that mediates self-destruction of the cell and so is often termed cellular suicide (Ameisen, 2002). PCD has been found to occur in all multicellular animals studied to date (reviewed by Jacobson *et al.*, 1997) and has been considered to be a feature unique to multicellular eukaryotes. Some evidence, however, suggests that PCD operates in some unicellular eukaryotes and in bacterial cells.

### 5.1.1 Programmed Cell Death in Eukaryotes

In multicellular organisms, PCD is classically known as apoptosis (Kerr *et al.*, 1972). Apoptosis functions to eliminate cells during development, maintains proper cell homeostasis by driving the constant turnover of cells, and eradicates defective cells (i.e. cancerous cells) and cells that are damaged by infection, chemicals or environmental factors and whose repair would be too costly to the system as a whole (Rice and Bayles, 2003). Thus, in multicellular organisms, PCD is 'altruistic', ensuring survival of an entire organism by programmatically eliminating a single or subpopulation of damaged cells.

Affecting a single eukaryotic cell, the process of apoptosis involves a complex network of regulators and effectors that mediate cellular destruction. Regardless of the initial stimulus, apoptotic cells exhibit a typical phenotype that includes cell shrinkage, chromatin condensation, proteolysis, cytoplasmic 'blebbing' and vacuolisation and DNA degradation (Hochman, 1997). Following this, the cell is fragmented into distinct apoptotic bodies that are phagocytosed by specialised cells of the immune system or by the surrounding tissue. This distinctive morphology has been reported to occur during the death of unicellular organisms, suggesting that PCD is not unique to multicellular species.

As early as 1996, various forms of PCD had been described in four different species of unicellular eukaryotes, including two species of the parasitic *Trypanosoma* (Ameisen *et al.*, 1995; Welburn *et al.*, 1996), the free-living slime mould *Dictyostelium discoideum* (Cornillon *et al.*, 1994), and the free-living ciliate *Tetrahymena thermophila* (Christensen *et al.*, 1995). These unicellular organisms have been found to die with many features characteristic of apoptosis in higher organisms, including DNA fragmentation, chromatin condensation and cytoplasmic blebbing and vacuolisation. The death process may be regulated by extracellular signals or environmental stress, such as starvation or oxidative damage, which is further analogous to apoptosis. Other species have since been reported to exhibit an apoptosis-like death phenotype, including three species of *Leishmania* (Moreira *et al.*, 1996; Das *et al.*, 2001; Arnoult *et al.*, 2002), the chlorophyte algae *Dunaliella tertiolecta* (Segovia *et al.*, 2003) and the dinoflagellate *Peridinium gatunense* (Vardi *et al.*, 1999).

Yeasts may also die by a PCD mechanism. In a study by Madeo *et al.* (1999), the application of a lethal dose of hydrogen peroxide to *Saccharomyces cerevisiae*

caused morphological changes that closely resembled apoptosis of metazoan cells. These included condensation of chromatin, DNA fragmentation and membrane permeabilisation. Further, expression of mammalian pro- or anti-apoptotic proteins in yeast cells induce or prevent death (Hanada *et al.*, 1995). More direct evidence for the occurrence of PCD in yeast cells was reported by Madeo *et al.* (2002). That group identified a specific apoptosis-mediating protein in *S. cerevisiae*, which is activated by hydrogen peroxide or ageing.

### **5.1.2 Bacterial Programmed Cell Death**

Based on the definition of PCD as a genetically determined, self-mediated process of cell death, several developmental processes in bacteria, such as sporulation and fruiting body formation, have been argued to involve primitive forms of PCD (reviewed by Lewis, 2000). In the sporulating *Bacillus subtilis*, the spore is released following death of the mother cell, which is actively lysed by autolysins synthesised by the cell itself (Smith and Foster, 1995; Nugroho *et al.*, 1999). This process involves a cascade of events and over 60 genes encoded on the chromosome of *B. subtilis* (Losick *et al.*, 1986; Errington, 1993). Normal fruiting body formation and sporulation in *Myxococcus xanthus* requires 'autocides', which include fatty acids and glucosamine, that induce autolysis of the cell (Rosenbluh and Rosenberg, 1989; Rosenbluh and Rosenberg, 1990; Mueller and Dworkin, 1991). Although the exact role of this autolysis is not clear, the release of nutrients from the killed cells probably feeds the fruiting body and developing spores. In *Streptomyces*, the death of a large number of hyphae during its life cycle shares some features with eukaryotic apoptosis such as genomic DNA fragmentation and membrane blebbing (Migueluez *et al.*, 1999), suggesting that this hyphal death is genetically programmed.

In fact, apoptosis-like phenotypes have been observed during cell death of a number of bacteria (Kusters *et al.*, 1997; Cellini *et al.*, 2001; Ning *et al.*, 2002; Berman-Frank *et al.*, 2004), providing some indirect evidence for the existence of PCD in certain species of bacteria. In addition to the occurrence of bacterial PCD during developmental processes, a considerable amount of evidence for PCD of defective or damaged cells has been reported in the literature (reviewed by Lewis, 2000).

### **5.1.3 Toxin-Antitoxin Systems**

The identification of a group of genetic elements able to mediate cell death in bacteria has provided more direct, genetic-based evidence for the existence of PCD in prokaryotes. These elements encode for two components, one being lethal to the cell and the other suppressing that toxic effect and so are called toxin-antitoxin (TA) systems (Engelberg-Kulka and Glaser, 1999; Gerdes, 2000). Although TA systems are ubiquitous amongst bacteria (Gotfredsen and Gerdes, 1998; Grönlund and Gerdes, 1999; Grady and Hayes, 2003) they are described below in context to *E. coli* only, because this is of relevance to the current work. It should be noted, however, that these systems are certainly not unique to *E. coli*. Table 5.1 is a non-exhaustive list of TA systems, and their functional components, identified in *E. coli*.

The TA module generally consists of two genes; the product of the second gene is a stable protein that is lethal to the cell (the toxin), whereas the first gene encodes a labile protein or antisense RNA molecule that inhibits the effect of the toxin (the antitoxin, or antidote). The only exception to this genetic organisation is the *hig* locus of the plasmid Rts1 of *E. coli*, where the first cistron encodes for the toxin (Tian *et al.*, 1996).

**Table 5.1** Major *E. coli* toxin-antitoxin systems. Adapted from Gerdes *et al.* (1997), Couturier *et al.* (1998), Engelberg-Kulka and Glaser (1999) and Hayes (2003).

| TA system                                 | Other nomenclature  | Location             | Toxin | Antitoxin <sup>a</sup> | Target of toxin | Antitoxin degradation |
|---|---------------------|----------------------|-------|------------------------|-----------------|-----------------------|
| <b>Extrachromosomally-encoded systems</b> |                     |                      |       |                        |                 |                       |
| <i>ccd</i>                                | H/G; <i>letAB</i>   | F plasmid            | CcdB  | CcdA                   | Gyrase A        | Lon                   |
| <i>pem/parD</i>                           | <i>kis/ kid</i>     | Plasmid R100/R1      | PemK  | PemI                   | DnaB            | Lon                   |
| <i>parDE</i>                              | -                   | Plasmid RK2/RP4      | ParE  | ParD                   | Unknown         | Unknown               |
| <i>phd-doc</i>                            | -                   | Prophage P1          | Doc   | Phd                    | Unknown         | ClpXP                 |
| <i>relBE</i>                              | -                   | Plasmid P307         | RelE  | RelB                   | Unknown         | Lon                   |
| <i>hig</i>                                | -                   | Plasmid Rts1         | HigB  | HigA                   | Unknown         | Unknown               |
| Hok/Sok family <sup>b</sup>               | -                   | Plasmid R1/F plasmid | Hok   | Sok <sup>c</sup>       | Unknown         | RNaseE                |
| <b>Chromosomally-encoded systems</b>      |                     |                      |       |                        |                 |                       |
| <i>mazEF</i>                              | <i>chpA; chpAIK</i> | <i>E. coli</i>       | MazF  | MazE                   | Unknown         | ClpPA                 |
| <i>chpB</i>                               | -                   | <i>E. coli</i>       | ChpBK | ChpBI                  | Unknown         | ClpPA                 |
| <i>relBE</i>                              | -                   | <i>E. coli</i>       | RelE  | RelB                   | Unknown         | Lon                   |
| <i>yefM-yoeB</i>                          | -                   | <i>E. coli</i>       | YoeB  | YefM                   | Unknown         | Lon?                  |
| <i>dinJ-yafQ</i>                          | -                   | <i>E. coli</i>       | YafQ  | DinJ                   | Unknown         | Unknown               |

<sup>a</sup> Protein molecule unless otherwise stated.

- None.

<sup>b</sup> Includes *hok/sok* of plasmid R1 and *flm* and *srn* systems of the F plasmid.

<sup>c</sup> Antitoxin is antisense RNA molecule.

? Not confirmed.

The proteic toxins are very effective lethal agents. Their artificial overproduction causes rapid cell killing of a substantial level, generally corresponding to a loss in cell viability of several orders of magnitude (Aizenman *et al.*, 1996; Gotfredsen and Gerdes, 1998; Grady and Hayes, 2003). The toxin presumably kills the cell by targeting a cellular component that is essential for cell survival and inhibiting its activity; however in all except a few cases the actual toxin target remains to be identified. Bernard and Couturier (1992), through the genetic analysis of *E. coli* mutants resistant to the toxic effect of CcdB of the *ccd* TA system, determined its cellular target to be DNA gyrase. In that study, mutations were mapped to the *gyrA* gene that encodes for GyrA, the catalytic subunit of DNA gyrase. DNA gyrase is an essential enzyme that catalyses the ATP-dependent negative supercoiling of DNA and facilitates replication, transcription, transposition and various recombination reactions (Bernard and Couturier, 1992). Biochemical studies have indicated that binding of CcdB to the GyrA subunit inhibits the supercoiling activity of DNA gyrase (Maki *et al.*, 1992), leads to increased DNA cleavage (Bernard and Couturier, 1992) and interferes with the passage of RNA polymerases along the DNA (Critchlow *et al.*, 1997). Whether one or all of these events can account for cell death *in vivo* is unclear (Engelberg-Kulka and Glaser, 1999). The only other known toxin target is DnaB that appears to be inhibited by the PemK toxin of the *pem* TA system to prevent DNA replication (Ruiz-Echevarría *et al.*, 1995).

The antitoxin component of a TA system inhibits the lethal effect of the toxin in one of two ways, depending on its form. Where the product of the antitoxin gene is a protein, the toxin and antitoxin are coexpressed and the antitoxin forms a tight complex with the toxin to antagonise its activity. The formation of these complexes has been directly shown for the *ccd* (Tam and Kline, 1989), *pem* (Ruiz-Echevarría *et al.*, 1995), *phd/doc* (Gazit and Sauer, 1999), *hig* (Tian *et al.*, 2001), *parDE* (Johnson

*et al.*, 1996) and *mazEF* (Aizenman *et al.*, 1996; Kamada *et al.*, 2003) TA pairs. It is presumed that this binding prevents subsequent binding of the toxin to its cellular target. While the majority of TA pairs encode for a proteic antitoxin, some encode instead for an antisense RNA molecule, including the *hok/sok* system of plasmid R1 and two TA pairs (*fim* and *srm*) on the F plasmid (reviewed by Gerdes *et al.*, 1997). In these incidences, the antisense RNA molecules prevent translation of the toxin-encoding mRNA and therefore the toxic protein is not produced when the antitoxin is present.

In addition to antagonising the effect of its associated toxin, the proteic antitoxin has a role in regulating the expression of the TA system. The antitoxin, either on its own or when bound to its associated toxin, negatively autoregulates transcription of the TA pair by binding to the promoter region(s) (Tam and Kline, 1989; Ruiz-Echevarría *et al.*, 1991; Magnuson *et al.*, 1996; Marianovsky *et al.*, 2001). The *ccd* locus of the F plasmid is unique in that both the CcdA and CcdB proteins are required to autoregulate transcription because CcdA has no repressor activity on its own (Tam and Kline, 1989).

The antitoxin is normally present in the cell at higher concentrations than its cognate toxin however, unlike the stable toxin, the proteic antitoxin is labile and is degraded by a specific protease, such as Lon or Clp (Engelberg-Kulka and Glaser, 1999); and the antisense RNA antitoxin (e.g. Sok) is degraded by a nuclease (e.g. ribonucleaseE) (Couturier *et al.*, 1998). The cellular proteases probably recognise the antitoxins in both their free form and when bound to their associated toxin, or the TA complex may dissociate at a rate which allows the free antitoxin to be degraded (Gerdes, 2000). This instability of the antitoxin is the basis for the activation of the toxin, or rather for the loss of toxin inhibition.

### **5.1.3.1 Plasmid-Encoded Toxin-Antitoxin Systems**

TA loci have been identified predominantly on extrachromosomal units, such as plasmids, where they are responsible for maintenance of that plasmid in the bacterial population. Upon its formation, a daughter cell inherits a pool of toxin-antitoxin complexes as well as free antitoxin molecules. If the cell is cured (i.e. lacks the plasmid), neither the toxin nor antitoxin pools are replenished and, because the antitoxin is degraded far more rapidly than the toxin due to the action of a specific protease, the toxin becomes available to exert its lethal action and the cell is killed (Jensen and Gerdes, 1995). TA pairs encoded by parasitic elements have been called 'addiction modules' because the cell is said to be addicted to the *de novo* synthesis of the antitoxin, which is essential for the cell's survival (Yarmolinsky, 1995). The programmatic removal of defective cells by plasmid-encoded TA modules is known as the post-segregational killing effect and, along with other mechanisms, is responsible for the maintenance of extrachromosomal units in the bacterial population (Gerdes *et al.*, 1986).

In *E. coli*, more than one addiction module may be encoded by a single plasmid. For example, the F plasmid encodes the *ccd*, *flm* and *srn* addiction modules and plasmid R1 includes the *hok/sok* and *pem* loci. Although all are considered to be responsible for post-segregational killing of plasmid-free cells, these systems function independently of each other and differ in their effectiveness as killers of cured cells (Jensen and Gerdes, 1995). It is not entirely clear why multiple addiction modules exist on a single extrachromosomal unit.

### **5.1.3.2 Chromosomally-Encoded Toxin-Antitoxin Systems**

The *E. coli* chromosome encodes a number of TA loci that are homologous to plasmid-encoded TA systems (see Table 5.1). They operate similarly to the

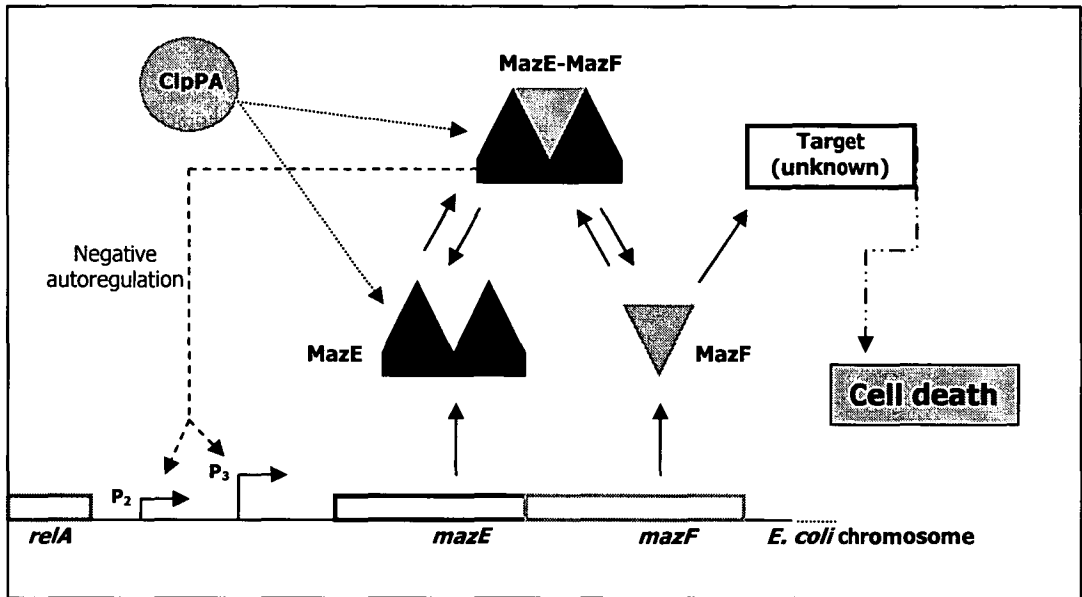


plasmid-encoded addiction modules described above but may occur for a much different reason. This reason has not been definitively determined although a major source of information has come from studies of the *mazEFTA* module.

### *mazEF*

The *mazEFTA* locus, also known as *chpA* or *chpAIK*, was first identified by Masuda *et al.* (1993) based on its homology to the *pem* and *parD* addiction modules of plasmids R100 and R1 respectively. Interestingly, MazE, the antitoxin of the *mazEF* TA system, not only antagonises the toxin affect of MazF but also of the *pem* and *parD* toxins PemK and Kid, indicating that there may be cross-talk between homologous TA loci (Santos-Sierra *et al.*, 1997; Santos-Sierra *et al.*, 1998). The *mazEF* module is located on the *E. coli* chromosome within the *rel* operon and downstream of the *relA* gene (Masuda *et al.*, 1993). The *mazE* cistron encodes for an 82 amino acid, 9.4 kDa proteic antitoxin that is coexpressed with the 111 amino acid, 12.1 kDa toxin encoded by the downstream *mazF* gene (Masuda *et al.*, 1993).

Aizenman *et al.* (1996) were the first to describe the *mazEF* system as a chromosomally-encoded TA module in *E. coli*. Through a series of experiments, they established that MazF is toxic and MazE its antitoxin; MazF is stable while MazE is a labile protein degraded *in vivo* by the ClpPA protease; MazE and MazF interact and are coexpressed; and the two *mazEF* promoters are strongly and negatively autoregulated by the combined action of MazE and MazF (Marianovsky *et al.*, 2001). Aizenman *et al.* (1996) concluded that *mazEF* has all the properties required for a TA module and thereby constitutes the first known chromosomally-encoded TA system. The *mazEF* genes and functional components are represented diagrammatically in Figure 5.1.



**Figure 5.1** Genetic organisation and functional components of the chromosomally-encoded *mazEF* TA module in *E. coli*. The protein products of the *mazE* and *mazF* genes interact to negatively autoregulate transcription at the  $P_2$  and  $P_3$  promoter sites. The serine protease ClpPA degrades MazE, allowing MazF to target an unknown molecule, which leads to death of the cell. Adapted from Gerdes (2000).

The artificial overexpression of MazF inhibits cell growth (Masuda *et al.*, 1993) and cell viability (Aizenman *et al.*, 1996). The cellular target of MazF has not been elucidated; however overexpression of the toxin *in vivo* has been shown to inhibit translation by inducing cleavage of mRNA molecules (Christensen *et al.*, 2003). The mechanism of this cleavage is not well understood due to conflicting results of the RNA cleavage specificity of MazF (Christensen *et al.*, 2003; Zhang *et al.*, 2003; Muñoz-Gómez *et al.*, 2004).

In addition to the above-mentioned properties that are characteristic of TA modules, a feature that is unique to the *mazEF* system has been identified (Aizenman *et al.*, 1996). Aizenman *et al.* (1996) reported that *mazEF* expression is inhibited by high concentrations of guanosine-3',5'-bispyrophosphate (ppGpp), a molecule that

reaches high cellular concentrations following nutritional starvation (Cashel and Gallant, 1969). Aizenman *et al.* (1996) proposed a model in which nutritional starvation causes increased levels of ppGpp. In the incidence of amino acid starvation, this increase in ppGpp concentration involves the product of the *relA* gene, which is located within the same operon as *mazEF*. The subsequent ppGpp levels inhibit the expression of *mazEF* and due to the labile nature of MazE, its cellular concentration decreases, allowing MazF to exert its toxic effect and kill the cell. Thus, that group have postulated that the *mazEF* mediates PCD in response to extreme nutritional stress.

Further experiments have demonstrated that the artificial overproduction of ppGpp induces *mazEF*-mediated cell death, supporting the above model (Aizenman *et al.*, 1996; Engelberg-Kulka *et al.*, 1998). ppGpp was induced by two means; firstly, by using a truncated *relA* gene under the control of an IPTG-inducible promoter (Aizenman *et al.*, 1996) and then by adding serine hydroxamate to the growth medium of a *relA*<sup>+</sup> *E. coli* strain to cause amino acid starvation (Engelberg-Kulka *et al.*, 1998). Using both methods, approximately 90% of the population were killed following the rapid induction of ppGpp. When the strain was deleted for *mazEF* or mutated to *clpP*, only 40% of the cells were killed, indicating that this process is *mazEF*-mediated and dependent on *clpP*. Gerdes (2000) has cautioned against the use of the truncated *relA* method and has not observed cell killing during starvation induced by the addition of serine hydroxamate. Further, that group have reported that rather than being inhibited by amino acid starvation, *mazEF* transcription is induced (Christensen *et al.*, 2003).

Despite this, that *mazEF* may mediate cell death in response to nutritional starvation has been further supported by the finding that *mazEF* appears to mediate cell death

when cells are starved for thymine (Sat *et al.*, 2003). That study used an *E. coli* strain containing a *mazEF* deletion, which remained almost 100% viable under conditions of thymine starvation whereas over 90% of the wild-type strain was killed. In addition, the activity of the *mazEF* P<sub>2</sub> promoter, which is primarily responsible for transcription of *mazEF* (Marianovsky *et al.*, 2001), was greatly reduced under conditions of thymine starvation (Sat *et al.*, 2003).

As well as being triggered by nutritional stress, *mazEF*-mediated cell death appears to be triggered by stressful conditions that inhibit the expression of the *mazEF* system. The antibiotics rifampin, chloramphenicol and spectinomycin, which are general inhibitors of transcription and translation, have been directly implicated as triggers of *mazEF*-mediated cell killing (Sat *et al.*, 2001). The Doc protein of the *phd/doc* addiction module is also a general inhibitor of translation and triggers cell death via the *mazEF* TA pair (Hazan *et al.*, 2001). It appears that by non-specifically inhibiting expression of the labile MazE protein, these molecules induce MazF toxicity. Furthermore, *mazEF*-mediated cell death is triggered by oxidative stress, treatment with high temperature (50°C), and exposure to the DNA-damaging agents mitomycin C, nalidixic acid and UV irradiation (Hazan *et al.*, 2004). Whether other stressful conditions that cause death of *E. coli*, such as low  $a_w$ , involve *mazEF* remains to be determined.

#### *Other Chromosomally-Encoded Systems*

At least four other TA systems are encoded on the *E. coli* chromosome (see Table 5.1); three (*relBE*, *yefM-yoeB* and *dinJ-yafQ*) are homologous to the *relBE* addiction module on plasmid P307 and the fourth (*chpB*) is a homologue of *mazEF*. All of these systems function similarly to the *mazEF* module and so will not be described in detail here. In addition to these prototypical TA systems, the *E. coli* chromosome

might encode other TA modules. Numerous homologues of the *mazEF* (Gerdes, 2000), *relBE* (Gotfredsen and Gerdes, 1998; Grönlund and Gerdes, 1999) and *hok/sok* (Pedersen and Gerdes, 1999) TA modules have been described. Also, cell death mechanisms that do not show homology to known TA loci but exhibit features characteristic of TA modules have been reported (Bishop *et al.*, 1998; Brown and Shaw, 2003).

Why multiple, and homologous, TA systems are present on a single chromosome is not known, however a report by Christensen *et al.* (2003) suggests that some differences between these loci may account for the apparent redundancy of genes. That group showed that MazF and RelE cleaved RNA during amino acid starvation but ChpBK did not. Therefore, different triggers may induce different TA loci and some TA systems may mediate cell death only under particular physiological states, such as carbon or amino acid starvation; and stationary or exponential phase (Christensen *et al.*, 2004). Furthermore, MazF and ChpBK show different RNA cleavage patterns (Christensen *et al.*, 2003), suggesting that each toxin may target separate subsets of mRNA.

#### **5.1.4 Role of Toxin-Antitoxin Systems**

Plasmid-encoded addiction modules function to maintain the plasmid within the bacterial population via post-segregational killing and this function has been well established (Jensen and Gerdes, 1995). In direct contrast, the role of chromosomally-encoded TA modules is unclear. When artificially encoded on plasmids, many of the normally chromosomally-encoded TA modules are able to stabilise plasmids just as effectively as their plasmid-encoded homologues (Gotfredsen and Gerdes, 1998; Grönlund and Gerdes, 1999). Although some

chromosomally-encoded TA modules have been reported to mediate post-segregational killing (Hazan *et al.*, 2001), this does not appear to be their primary function (Gerdes, 2000).

For an individual bacterial cell, the presence of a TA module on the chromosome that acts as a PCD system appears to be counterproductive, much like apoptosis in individual prokaryotic cells. However, growing experimental evidence suggests that bacterial populations act as multicellular organisms (reviewed by Shapiro, 1988; Shapiro, 1998; Krasovec and Jerman, 2003; Pray and Anderson, 2003; Webb *et al.*, 2003), and in this respect, the existence of chromosomally-encoded TA systems may be of beneficial value to the population. A number of potential benefits have been proposed although none have been definitively proven either experimentally or theoretically.

In response to nutritional stress, the programmed death of part of the population reduces competition for available nutrients and may allow neighbouring cells to survive or even grow on nutrients that are released from the dead cell upon its lysis (Aizenman *et al.*, 1996). The 'altruistic' death of a subpopulation of cells in response to starvation or other stresses leads to the development of 'persistors', which presumably survive because they lack the TA system or it is atypical (Lewis, 2000; Aertsen and Michiels, 2004). Alternatively, chromosomally-encoded TA systems may provide a defence mechanism against the spread of phages within the population. The ability of the *mazEF* system to mediate cell death in *E. coli* and thereby inhibit the spread of prophage P1 has been experimentally shown by Hazan and Engelberg-Kulka (2004). Chromosomally-encoded TA systems may also be utilised by the bacterial population to maintain genomic stability by systematically removing cells whose genome or other key systems are damaged. The finding that

DNA-damaging agents can trigger *mazEF*-mediated cell death supports this idea (Hazan *et al.*, 2004).

Establishing the function of chromosomally-encoded TA pairs has been complicated by findings that at least some of these systems, namely the *mazEF* and *relBE* modules, cause a bacteriostatic condition rather than mediating cell death. Pedersen *et al.* (2002) used *E. coli* strains deleted for the chromosomal *relBE* module, and found that the overexpression of RelE to high levels (by inducing transcription of *relE* encoded on a plasmid) caused a drastic loss in cell viability of approximately  $6.0 \log \text{ CFU.mL}^{-1}$ . Importantly, the subsequent overexpression of RelB (induced using a separate plasmid) in cells transferred to LB plates reversed RelE toxicity. The ability of RelB to resuscitate these cells indicated that RelE did not kill the cells but instead forced them into a state in which they were permanently stationary and unable to form a colony. Similar results were found for the *mazEF* system (Pedersen *et al.*, 2002). That group postulated that rather than being toxins, chromosomally-encoded TA systems regulate global rates of protein synthesis during starvation or other stresses, thereby acting as growth modulators to protect the cell exposed to harsh environments (Gerdes, 2000; Pedersen *et al.*, 2002; Christensen *et al.*, 2003).

Amitai *et al.* (2004) repeated the above experiment, using similar plasmids and mutants absent for the chromosomally-encoded TA modules, and extended the timeframe of analysis from around five to 24 hours. While they confirmed that RelB and MazE could reverse the effect of their cognate toxins up until the five hour time point, those authors concluded that after approximately six hours of toxin expression, the ability of these antitoxins to resuscitate cells is lost. They,

therefore, suggest that there is a 'point of no return' and following this point RelE and MazF are indeed mediators of cell death.

Studies previously detailed in this thesis have provided a quantitative description of the inactivation of *E. coli* in response to osmotic stress. Although findings from that work have had useful applications in the production of safe foods (see Chapter 4), they have not significantly contributed to our understanding of the physiology of *E. coli* in declining populations. The aim of the current chapter was to begin to provide a mechanistic understanding of the actual process(es) involved in the low  $a_w$ -induced destruction of bacterial populations. Chromosomally-encoded TA systems are the first genes in *E. coli* to be directly implicated in cell death and were, therefore, considered prime candidates to mediate the death of *E. coli* in response to low  $a_w$  stress. Thus, the specific aim of the current chapter was to determine if the *mazEF* TA system mediates cell death in *E. coli* upon exposure to low  $a_w$ . Further, the timing of any *mazEF*-mediated cell killing was assessed and related to the phases of inactivation (P1, P2 and P3) described in Section 3.4.1.

## **5.2 MATERIALS AND METHODS**

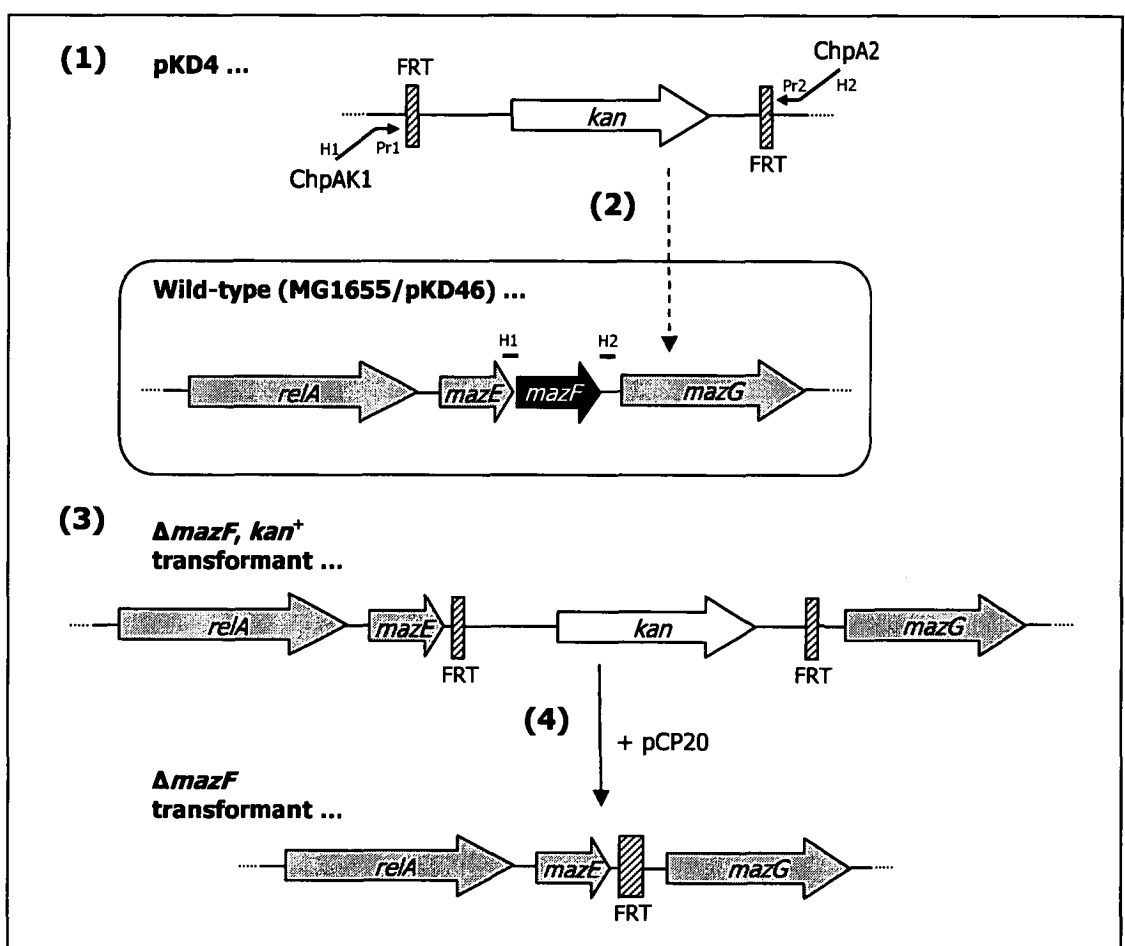
### **5.2.1 Bacterial Strains, Reagents, Media, Solutions and Equipment**

Details of bacterial strains, chemical reagents, bacteriological media, solutions and equipment are shown in Appendix A. Construction of the  $\Delta mazF$  mutant (SM01) used *E. coli* MG1655/pKD46 as the parental strain and *E. coli* N1 as an intermediate strain. Other strains were used to maintain plasmids. The  $\Delta mazF$  *E. coli* mutants (SM01 and SC30) and their wild-type (*E. coli* MG1655) were used in subsequent experiments to confirm proper construction of the mutant and to assess the effect of *mazF* on the inactivation of *E. coli* MG1655 in response to various stresses.



### 5.2.2 Construction of the $\Delta mazF$ *Escherichia coli* Mutant (Strain SM01)

The procedure used to construct a  $\Delta mazF$  *E. coli* mutant (strain SM01) was adapted from Datsenko and Wanner (2000) for which a schematic representation is given in Figure 5.2. Only the *mazF* gene (that encodes for the proteic toxin MazF) was deleted, as opposed to the entire *mazEF* module that also includes *mazE*, the antitoxin-encoding gene. Unless otherwise stated, all centrifugation steps were at 13 000 rpm in a Hettich Zentrifugen EBA12 centrifuge operating at RT.



**Figure 5.2** The procedure used to construct a  $\Delta mazF$  *E. coli* mutant. (1) PCR amplification of the Flp recognition target (FRT)-flanked kanamycin resistance gene (*kan*) from the template plasmid pKD4, using primers ChpAK1 and ChpA2 that include priming sites to pKD4 (Pr1 and Pr2 respectively) and homology extensions to the *E. coli* genome (H1 and H2 respectively). (2) Transformation of MG1655 expressing  $\lambda$  Red recombinase (from plasmid pKD46) with DNA fragment from Step 1. (3) Selection of kanamycin resistant transformants. (4) Elimination of resistance cassette using pCP20 (a Flp expression plasmid).

### 5.2.2.1 Primers and Plasmids

Table 5.2 lists the primers and Table 5.3 the plasmids used in the construction and analysis of the  $\Delta mazF$  *E. coli* mutant. Plasmid pKD4 was maintained in *E. coli* BW25141, plasmid pKD46 in *E. coli* MG1655 and plasmid pCP20 in the *E. coli* DH5 $\alpha$  strain designated BT340.

**Table 5.2** Primers employed in the construction and analysis of the  $\Delta mazF$  *E. coli* mutant.

| Primer              | Sequence   | Size (bases) |
|---------------------|--|--------------|
| ChpA2 <sup>a</sup>  | 5'-ggg tct gtc agg tgg aaa cct gtg acc aga ata gaa gtg agt tag taa<br>cac ata tga ata tcc tcc tta g-3' | 70           |
| ChpAK1 <sup>a</sup> | 5'-cac gag aat atc gac tgg gga gag ccg aaa gat aag gaa gtc tgg<br>taa gtg tag gct gga gct gct tc-3'    | 68           |
| ChpR_F              | 5'-atg atc cac agt agc gta-3'  | 18           |
| k1                  | 5'-cag tca tag ccg aat agc ct-3'   | 20           |
| k2                  | 5'-cgg tgc cct gaa tga act gc-3'   | 20           |
| kt                  | 5'-cgg cca cag tcg atg aat cc-3'   | 20           |
| Relreg_F            | 5'-tcc tcc tcc agt tta g-3'  | 16           |
| Relreg_R            | 5'-ttg gca acc tga tgc-3'  | 15           |

<sup>a</sup> Underscored bases indicate the priming sites (Pr2 and Pr1 respectively) for the pKD4 template. The remaining bases are homology extensions (H2 and H1 respectively) incorporated to facilitate fusion of fragments in *E. coli*.

**Table 5.3** Plasmids used in the construction and analysis of the  $\Delta mazF$  *E. coli* mutant.

| Plasmid | Description (name)   | Size (kb) | Reference                         |
|---------|--|-----------|-----------------------------------|
| pKD4    | pANTSy, <i>bla</i> , FLP-flanked <i>kan</i> (template)   | 3.3       | Datsenko and Wanner (2000)        |
| pKD46   | pINT, <i>rep<sup>S</sup></i> , <i>bla</i> , arabinose-inducible $\lambda$<br>Red recombinase expression (red helper) | 6.3       | Datsenko and Wanner (2000)        |
| pCP20   | pSC101, <i>rep<sup>S</sup></i> , <i>bla</i> , <i>cat</i> (eliminator)  | 9.4       | Cherepanov and Wackernagel (1995) |

*bla*  $\beta$ -lactamase (ampicillin resistance) gene; *cat* chloramphenicol transacetylase (chloramphenicol resistance) gene; *kan* kanamycin resistance gene; *rep<sup>S</sup>* temperature-sensitive replicon.

### **5.2.2.2 General Methods**

#### *Plasmid Extraction*

Plasmid DNA was prepared using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Briefly, a single colony of *E. coli* BW25141/pKD4 or BT340 (carrying the eliminator plasmid pCP20) on TSA containing ampicillin (A.1.1) was transferred to 10 mL TSB containing ampicillin (A.1.3) and incubated overnight ( $18 \pm 2$  hours). For extraction of the pKD4 plasmid, a 5 mL volume of the cell culture was pelleted by centrifugation at 14 000 rpm for 10 minutes at RT in a Sorvall® SUPER T21 centrifuge and the pellet was resuspended in 250  $\mu$ L of Buffer P1. The cells were lysed by the addition of 250  $\mu$ L of Buffer P2 with gentle mixing and 350  $\mu$ L of Buffer N3 was added to neutralise the solution. The tube was centrifuged for 10 minutes and the supernatant transferred to a QIAprep column and centrifuged for 1 minute. The column was washed with 0.75 mL of Buffer PE and centrifuged for 1 minute. The flow-through was discarded and the spin repeated. 50  $\mu$ L Milli-Q H<sub>2</sub>O was applied to the column, left for 5 minutes and spun for 1 minute to elute the plasmid DNA. The pCP20 plasmid was extracted similarly except that a 30 mL volume of cell culture was pelleted and the volumes of Buffers P1, P2 and N3 were doubled. The purity of a 5  $\mu$ L aliquot of the plasmid DNA preparation was determined by agarose gel electrophoresis and the concentration of the sample quantified using a spectrophotometer.

#### *Agarose Gel Electrophoresis of DNA*

A 0.25 volume of loading buffer was added to an aliquot of DNA sample and the entire contents were loaded onto a 1% agarose gel. The DNA was separated by electrophoresis in a Horizon® 58 horizontal electrophoresis cell in 1X tris acetic acid EDTA buffer at 90 V for 0.5 – 1 hour. A molecular weight marker was run

simultaneously. The gel was visualised using an UV transilluminator with camera and the image was acquired using Kodak 1D software.

### *Polymerase Chain Reaction*

Prepared on ice, each PCR (20 or 50  $\mu$ L final volume) contained Hot Start Master Mix (10 or 25  $\mu$ L, containing PCR buffer with  $MgCl_2$ , *Taq* DNA polymerase and dNTPs), primers (2  $\mu$ L/primer of 10 pmol/ $\mu$ L stock), DNA template (5  $\mu$ L of cell suspension or 2  $\mu$ L of plasmid preparation) and Milli-Q  $H_2O$  (1 or 19  $\mu$ L to give a final volume of 20 or 50  $\mu$ L). The PCR cycle conditions were conducted in a thermocycler as follows:

|                     |                         |
|---------------------|-------------------------|
| 94°C for 15 minutes |                         |
| 94°C for 1 minute   | } Repeated for 30 times |
| 50°C for 1 minute   |                         |
| 72°C for 2 minutes  |                         |
| 72°C for 6 minutes  |                         |
| 10°C hold.          |                         |

### *Purification of the Polymerase Chain Reaction Product*

The PCR product was purified using the QIAquick Purification Kit and the accompanying instructions. Centrifugation was for 1 minute in all cases. Briefly, 1 volume of the PCR was mixed with 5 volumes of Buffer PB and the sample was applied to a QIAquick column and spun. The column was washed with 750  $\mu$ L of 35% guanidine hydrochloride, centrifuged and then washed with 0.75 mL Buffer PE and centrifuged twice. 50  $\mu$ L of Milli-Q  $H_2O$  was added to the tube and the tube was left to stand for 5 minutes and then centrifuged to elute the DNA.

### ***Preparation of Electrocompetent Cells and Electroporation with DNA***

Cells for electroporation were incubated at 4°C for 10 minutes and then pelleted by centrifugation at 4300 rpm for 10 minutes at 4°C in a Sorvall® SUPER T21 centrifuge. The cells were made electrocompetent by washing in ice-cold 10% glycerol twice (with centrifugation as described above) and were then resuspended in 50 µL of ice-cold 10% glycerol. A 40 µL volume of cells was gently mixed with 2.5 µL of DNA in an electroporation cuvette. The suspension was pulsed with 1.8 kV using an electroporator and 1 mL of recovery broth (A.1.3), pre-warmed to 37°C, was immediately added. The cell suspension was transferred to a sterile microcentrifuge tube and incubated at 37°C for 1 hour to recover. Competent cells with no DNA added were used as a negative control.

### ***5.2.2.3 Polymerase Chain Reaction Amplification of the Flp Recognition Target-Flanked Kanamycin Resistance Gene***

A 50 µL PCR was prepared (5.2.2.2) using primers ChpAK1 and ChpA2. The DNA template (~ 40 ng) was provided by plasmid pKD4 (5.2.2.2), at a concentration of approximately 20 ng/µL. A 5 µL aliquot was separated by gel electrophoresis (5.2.2.2) to confirm the reaction. The PCR product (25 µL) was purified (5.2.2.2) and a 5 µL aliquot was separated by gel electrophoresis (5.2.2.2) to determine purity. The purified DNA was quantified using a spectrophotometer.

### ***5.2.2.4 Gene Disruption***

A single colony of *E. coli* MG1655/pKD46 on TSA containing ampicillin (A.1.1) was transferred to 5 mL TSB containing ampicillin and L-arabinose (A.1.3) and incubated statically at 30°C for 16 hours. The cells were made electrocompetent and electroporated (5.2.2.2) with approximately 100 ng of the Flp recognition target (FRT)-flanked *kan* (i.e. the gene that encodes for kanamycin resistance) PCR

product (5.2.2.3), at a concentration of about 40 ng/μL. Half of the electroporated cell suspension was plated to TSA containing kanamycin (A.1.3) and incubated at 30°C for 24 hours to select for kanamycin resistant (Km<sup>R</sup>) transformants. Km<sup>R</sup> transformants were colony-purified on TSA at 37°C for 14 hours. To test for the loss of plasmid pKD46 from these transformants, the sensitivity of single colonies to ampicillin was determined by sub-culturing on TSA containing ampicillin (A.1.3) with incubation at 30°C for 24 hours. If the plasmid was not lost, a few colonies were colony-purified on TSA at 43°C for 16 hours and similarly tested.

#### **5.2.2.5 Verification of Insertion of the Kanamycin Resistance Gene**

To confirm that the intermediate mutant had the correct structure, the size of its PCR products were compared to that of the wild-type MG1655. Specifically, a Km<sup>R</sup>, pKD46<sup>-</sup> mutant on TSA (5.2.2.4) was subcultured on TSA containing kanamycin (A.1.3) and incubated at 37°C for 14 hours. *E. coli* MG1655 was isolated on TSA (A.1.1). Single colonies were transferred to 20 μL Milli-Q H<sub>2</sub>O using a sterile tip. Four 20 μL PCRs (5.2.2.2) were carried out using 5 μL of the cell suspension and primers Relreg\_F and ChpR\_F; kt and Relreg\_R; k1 and Relreg\_R; and k2 and Relreg\_F. A 5 μL aliquot of the PCR products were separated by gel electrophoresis (5.2.2.2) to determine the size of the products. The verified Km<sup>R</sup> intermediate mutant was designated *E. coli* N1.

#### **5.2.2.6 Elimination of the Kanamycin Resistance Gene**

Km<sup>R</sup>, pKD46<sup>-</sup> mutants were transformed with pCP20. Specifically, a single colony of *E. coli* N1 (5.2.2.5) on TSA (5.2.2.4) was colony-purified on TSA containing kanamycin (A.1.3) with incubation at 37°C for 14 hours. A single colony was transferred to 5 mL TSA containing kanamycin (A.1.3) and incubated statically at 37°C for 16 hours. The cells were made electrocompetent and electroporated

(5.2.2.2) with plasmid pCP20 (5.2.2.2), at a concentration of approximately 2 ng/μL. Half of the electroporated cell suspension was plated to TSA containing ampicillin (A.1.3) and incubated at 30°C for 24 hours. Single colonies were sub-cultured to TSA and incubated at 43°C for 16 hours and then subcultured to TSA with kanamycin or ampicillin (A.1.3) to confirm the loss of both antibiotic resistances.

#### **5.2.2.7 Verification of Elimination of the Kanamycin Resistance Gene**

To confirm that the mutant had the correct structure, the size of its PCR products were compared to that of the wild-type MG1655. Specifically, single colonies of a kanamycin- and ampicillin-sensitive mutant on TSA (5.2.2.7) and *E. coli* MG1655 on TSA (A.1.1) were transferred to 20 μL Milli-Q H<sub>2</sub>O using a sterile tip. Two 20 μL PCRs (5.2.2.2) were performed using 5 μL of the cell suspension and primers Relreg\_F and Relreg\_R; and kt and Relreg\_R. A 5 μL aliquot of the PCR products were separated by gel electrophoresis (5.2.2.2) to determine their size. The verified  $\Delta mazF$  *E. coli* mutant was named SM01.

#### **5.2.2.8 Sequence Analysis**

Ms. Sharee McCammon (Pers. comm., 2005) performed the analysis of *E. coli* SM01 described in this section. Single colonies of *E. coli* SM01 were sequenced immediately after the elimination of *kan* was verified (5.2.2.7). Single colonies of *E. coli* MG1655 and SC30 on TSA (A.1.1) were sequenced to provide a  $\Delta mazF$  negative and positive control respectively. Single colonies were transferred to 20 μL Milli-Q H<sub>2</sub>O using a sterile tip. A PCR was performed (5.2.2.2) using 5 μL of each cell suspension and primers Relreg\_F and Relreg\_R. The PCR products were purified (5.2.2.2) and quantified using a spectrophotometer. Each sequence reaction was prepared on ice and contained the purified PCR product (4 μL of 20 ng/μL stock), dideoxy terminator cycle sequencing mix (4 μL) and a primer (2 μL of 10 pmol/μL

stock of ChpR\_F or Relreg\_F). Reactions were performed in a thermocycler as follows:

|                     |   |                       |
|---------------------|---|-----------------------|
| 96°C for 20 seconds | } | Repeated for 30 times |
| 50°C for 20 seconds |   |                       |
| 60°C for 4 minutes  |   |                       |
| 10°C hold.          |   |                       |

The products were cleaned according to the manufacturer's instructions and then run on a sequencer. The sequence data was analysed using BioEdit software.

### **5.2.3 Evaluation of the Involvement of *mazEF* in the Inactivation of *Escherichia coli***

#### **5.2.3.1 Preparation of Exponential Phase Populations of *Escherichia coli* in Tryptone Soya Broth**

Exponential phase populations of *E. coli* (strains MG1655, SC30 and SM01) were prepared by transferring single colonies on TSA (A.1.1) to 80 mL TSB in a 250 mL Erlenmeyer flask and incubating statically at 37°C for 16 ( $\pm 0.25$ ) hours, to a population density of approximately 9.0 log CFU.mL<sup>-1</sup>. The populations were diluted 1/10<sup>7</sup> in 80 mL TSB in a 250 mL Erlenmeyer flask and incubated at 37°C until just turbid (typically 5.5 hours), which correlated to a population density of approximately 7.0 log CFU.mL<sup>-1</sup>. At this time, populations were diluted 1/10 000 in 80 mL TSB and incubated at 37°C until just turbid (typically 3.5 - 4.5 hours) providing a population density of approximately 7.0 log CFU.mL<sup>-1</sup>.



### **5.2.3.2 Preparation of Exponential Phase Populations of *Escherichia coli* in Minimal Medium**

Exponential phase populations of *E. coli* (strains MG1655 and SC30) were prepared according to the method described by Hazan *et al.* (2004). Specifically, single colonies on TSA (A.1.1) were transferred to 80 mL MM in a 250 mL Erlenmeyer flask and were incubated statically at 37°C for 16 ( $\pm$  0.25) hours, to a population density of approximately 9.0 log CFU.mL<sup>-1</sup>. The populations were diluted 1/1000 in 80 mL MM in a 250 mL Erlenmeyer flask and incubated at 37°C in a water bath with shaking at 60 oscillations per minute until just turbid (typically 5.0 hours). This correlated to a population density of approximately 7.0 log CFU.mL<sup>-1</sup>, which is the equivalent of an optical density of 0.4 at 600 nm (L. Mellefont, Pers. comm., 2005).

### **5.2.3.3 High Temperature Treatment**

Aliquots (1 mL) of exponential phase populations of *E. coli* MG1655 or SC30 prepared in TSB (5.2.3.1) were transferred to McCartney bottles containing 9.0 mL of TSB that had been equilibrated to the required temperature. Alternatively, 1 mL aliquots of exponential phase populations of *E. coli* MG1655 or SC30 prepared in MM (5.2.3.2) were transferred to McCartney bottles containing 9.0 mL of MM that had been equilibrated to the required temperature. In either case, a 1/10 dilution of the original exponential phase population was achieved. Flasks were incubated at the appropriate temperature in a water bath with shaking at 60 oscillations per minute. The temperature during each treatment was estimated by monitoring the temperature in a replicate broth using a temperature data logger set to read every 5 seconds and GLM software. The temperature was always within  $\pm$  0.2°C of the average value. The number of viable cells was determined by culture-based methodologies (2.2.2.4) immediately prior to the high temperature treatment, and either after 10.0 minutes of incubation or at regular intervals throughout (up to 18

minutes). When required, the percent survival of each population was calculated by subtracting the absolute number of viable cells (CFU.mL<sup>-1</sup>) from that immediately prior to inoculation and multiplying that number by 100. The percent survival (depicted on a logarithmic scale) was plotted vs. temperature of treatment. The data was analysed for significant differences ( $P \leq 0.01$ ) via multiple Student's *t*-tests (two-sample assuming equal variances) using Microsoft® Excel.

#### **5.2.3.4 Low Water Activity Treatment**

The  $a_w$  of TSB was reduced to the required level (0.95 or 0.90) by the addition of NaCl (typically 7 or 16% weight per volume respectively). Aliquots (9 mL) were transferred to McCartney bottles, which were autoclaved (121°C, 20 minutes) and then temperature-equilibrated to 25°C. Exponential phase populations of *E. coli* MG1655, SC30 or SM01 prepared in TSB (5.2.3.1) were inoculated to these broths by transferring a 1 mL aliquot to the low  $a_w$ -broth, thus providing a 1/10 dilution of the original inoculum. Flasks were incubated at 25°C in a water bath with shaking at 60 oscillations per minute. Immediately following inoculation, a 1 mL aliquot was withdrawn to measure the  $a_w$ . The number of viable cells was determined by culture-based methodologies (2.2.2.4) using BHA-P immediately prior to, and at regular intervals throughout, the low  $a_w$  treatment. When required, the percent survival of each population was determined as described in Section 5.2.2.3 and was plotted on a logarithmic scale vs. time of treatment using Microsoft® Excel.

#### **5.2.3.5 Characterising mazEF-Mediated Cell Death During Low Water Activity-Induced Inactivation**

Survival curves generated by the methodology described in Section 5.2.3.4 for populations of *E. coli* MG1655 and SM01 were analysed to estimate the duration, rate of inactivation and loss of viability (based on the first and last enumerations)

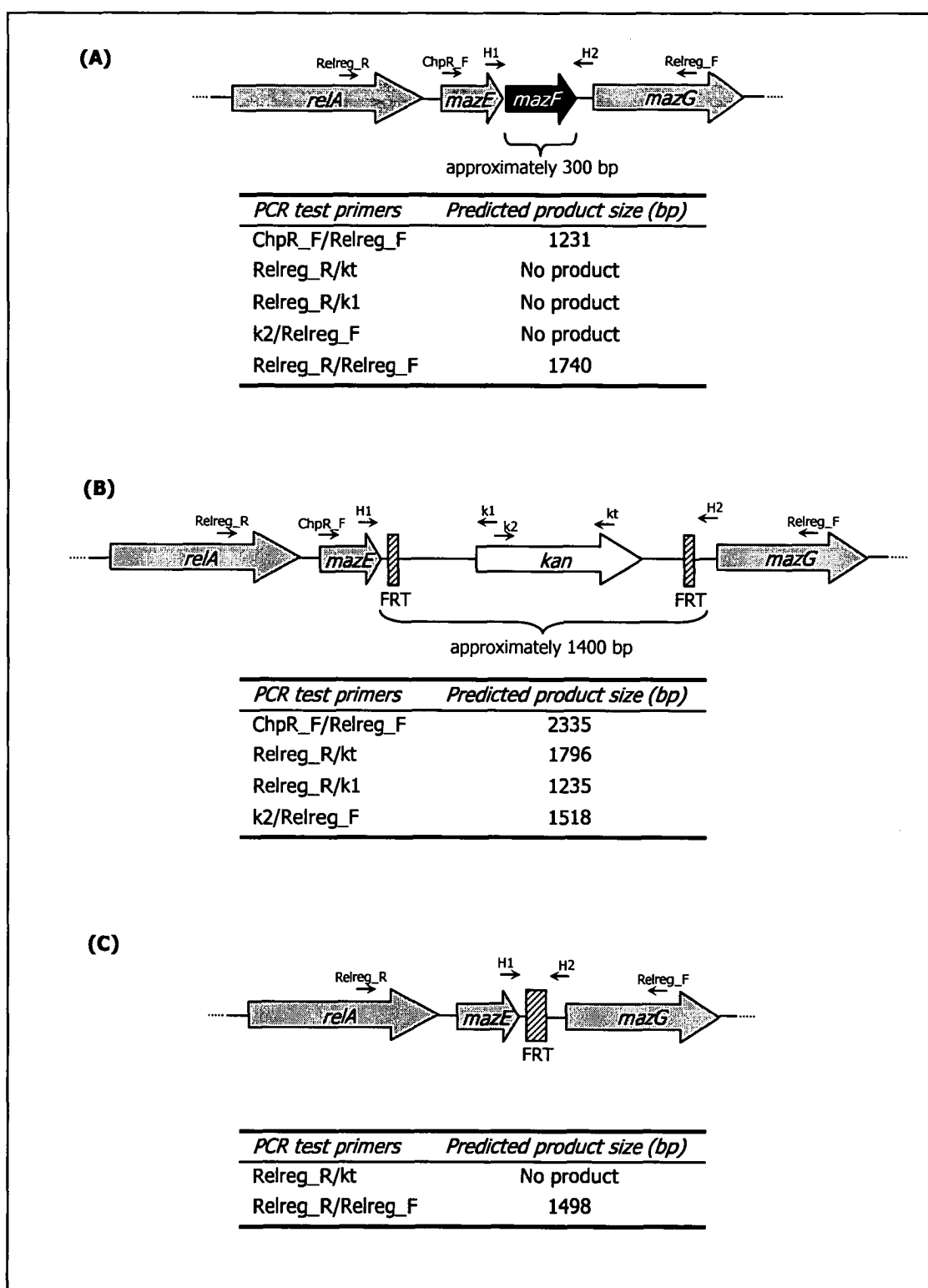
observed during each phase of inactivation. The differences in viable numbers during the second and third phases of inactivation were calculated by subtracting the log CFU.mL<sup>-1</sup> value of *E. coli* SM01 from that of *E. coli* MG1655 and plotted against time.

## 5.3 RESULTS

### 5.3.1 Construction of the $\Delta mazF$ *Escherichia coli* Mutant (Strain SM01)

A new mutant (*E. coli* SM01) deleted for *mazF*, the gene encoding the protein MazF, was constructed using the Red disruption system developed by Datsenko and Wanner (2000). The predicted structure of the *relA* operon (containing *mazF*) during the mutant's construction and the estimated sizes of its various DNA fragments are given in Figure 5.3.

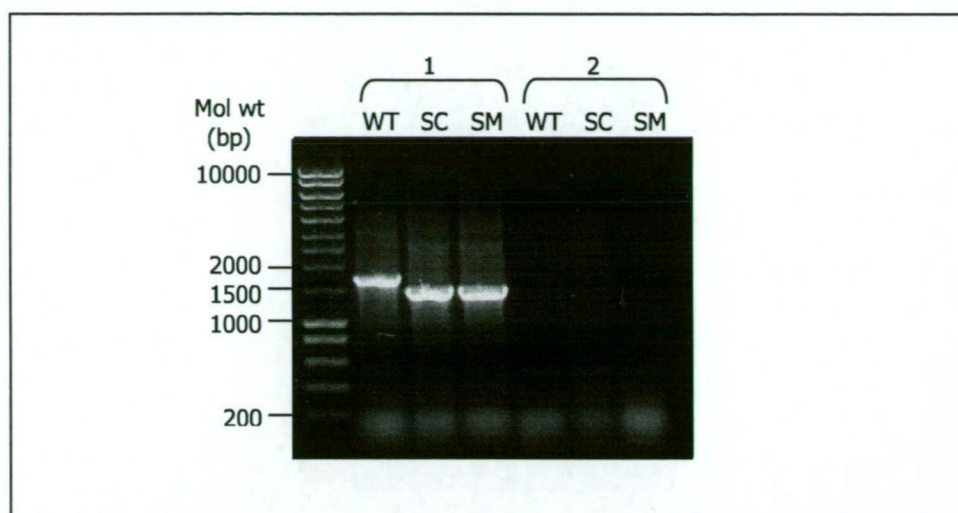
Specifically, the selectable kanamycin resistance gene (*kan*) was amplified by PCR from the template plasmid pKD4 using primers containing homology extensions to the targeted gene (*mazF*). Recombination of this fragment in *E. coli* MG1655 used the phage  $\lambda$  Red recombinase encoded by plasmid pKD46. The Red system includes three genes ( $\gamma$ ,  $\beta$  and *exo*) that encode for Gam, Bet and Exo. Gam inhibits host RecBCD exonuclease V, which allows Bet and Exo to access DNA ends to promote recombination (Datsenko and Wanner, 2000). One of the resulting Km<sup>R</sup> transformants (designated *E. coli* N1) was colony-purified and cured for plasmid pKD46 (a temperature sensitive replicon) by growing at 37°C.



**Figure 5.3** Predicted structure of the *relA* operon in (A) *E. coli* MG1655 (wild-type), (B) *E. coli* N1 (following disruption of *mazF* with the FRT-flanked *kan* fragment) and (C) *E. coli* SM01 (following elimination of *kan* by recombination of the FRTs). The primer-binding sites used during PCR verification and the predicted size (in bp) of the PCR test products are shown. H1 and H2 are homology extensions (see text for details).

Disruption of *mazF* in *E. coli* N1 was confirmed by size analysis of various PCR fragments (data not shown). Specifically, using *relA* operon-specific primers that bind near to *mazF* (i.e. ChpR\_F and Relreg\_F) and DNA template derived from *E. coli* N1 or the wild-type MG1655 (as a control), results indicated that *E. coli* N1 had lost the *mazF* (parental) fragment of ~ 300 bp and gained the ~ 1.4 kbp FRT-flanked *kan* fragment. Further, primers flanking *mazF* (i.e. Relreg\_R or Relreg\_F) were employed with the appropriate *kan*-specific primers (i.e. kt, k1 or k2) to test for the junction fragments. DNA fragments were produced in reactions containing *E. coli* N1 as the DNA template but not the wild-type strain. Collectively these results reveal that *E. coli* N1 contains the predicted junction and locus-specific fragments of the expected sizes.

Following confirmation of disruption to *mazF* in *E. coli* N1, the resistance gene was eliminated by transformation of that strain with plasmid pCP20 that encodes for Flp recombinase. This enzyme acts on directly repeated FRT sites to cause their recombination and thereby eliminates any chromosomal sequence between those positions. Following selection of ampicillin resistant (and therefore pCP20<sup>+</sup>) transformants, colonies were cured of plasmid pCP20, a temperature-sensitive replicon, by growth at 37°C. A single transformant (designated *E. coli* SM01) was analysed to confirm that the FRT-flanked resistance gene had been eliminated. PCRs were conducted using primer pairs Relreg\_F/Relreg\_R and Relreg\_R/kt and *E. coli* strains MG1655, SM01 and SC30 (a  $\Delta mazF$  mutant used as a positive control) as DNA templates. The DNA fragments generated by these PCRs are shown in Figure 5.4.



**Figure 5.4** DNA fragments generated using (1) operon-specific primers (Relreg\_R/Relreg\_F) and (2) a *mazF*-flanking primer (Relreg\_R) with a *kan*-specific primer (kt). DNA template used *E. coli* MG1655 (WT; wild-type), *E. coli* SC30 (SC; MG1655,  $\Delta mazF$ ) or *E. coli* SM01 (SM; MG1655,  $\Delta mazF$  transformant). Molecular weight markers were run simultaneously and are indicated on the left.

The PCR using *relA* operon-specific primers Relreg\_F and Relreg\_F and DNA template derived from *E. coli* strains SM01 and SC30 yielded DNA fragments of  $\sim 1.5$  kbp. Use of the same primer pair and the wild-type strain as the DNA template yielded a PCR product of  $\sim 1.7$  kbp. These results suggest that *E. coli* SM01 has lost the *kan* fragment of  $\sim 1.3$  kbp while retaining the FRT sites. *E. coli* SC30 provided a positive control for this procedure. No DNA product was generated from the PCRs that used primers Relreg\_R and kt and any of the *E. coli* strains tested and further confirmed that the *kan* fragment has been lost from *E. coli* SM01.

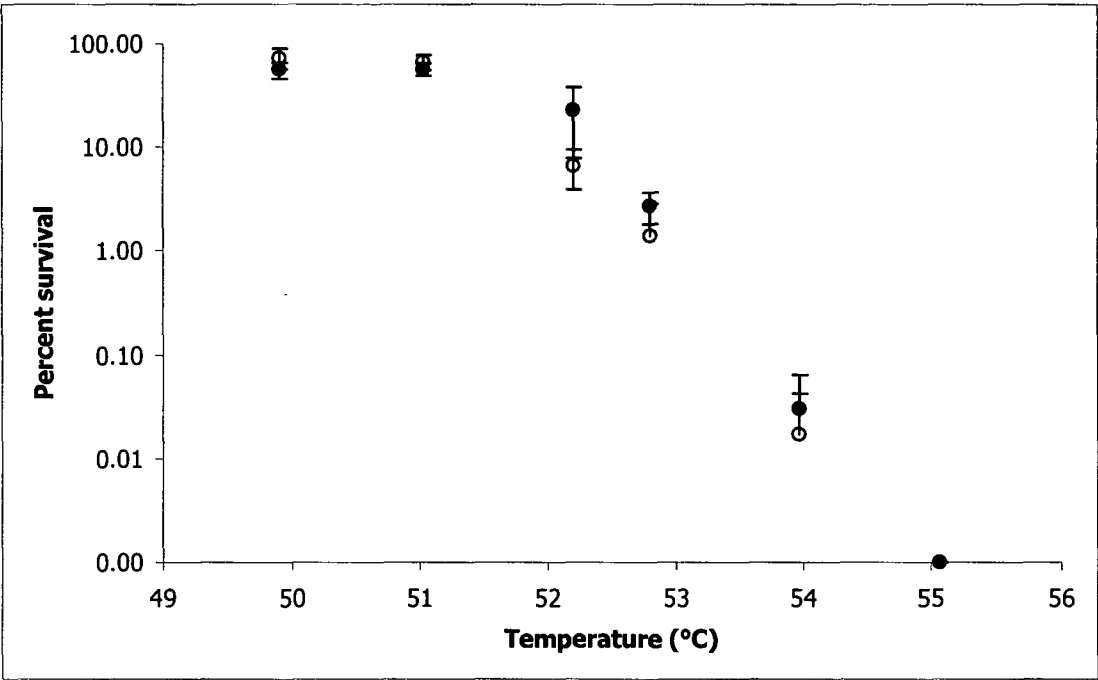
To confirm that *E. coli* SM01 had the desired deletion of *mazF*, the appropriate region of this strain was sequenced by Ms. Sharee McCammon (Pers. comm., 2005) immediately following its construction. Sequencing of *E. coli* SC30 and MG1655 provided positive and negative controls respectively. Results (data not shown)

indicate that the *mazF* gene is absent from *E. coli* SM01 and SC30 but present in *E. coli* MG1655. The flanking genes *mazE* and *mazG* were present in all strains. The scars remaining at the *mazF* location in *E. coli* SM01 and SC30 consist of the FRT sites and other bases. Thus the mutant generated in the current work (*E. coli* SM01) has the desired structure. This work further confirmed deletion of *mazF* in *E. coli* SC30.

### **5.3.2 Evaluation of the Involvement of *mazEF* in the Inactivation of *Escherichia coli***

#### **5.3.2.1 High Temperature**

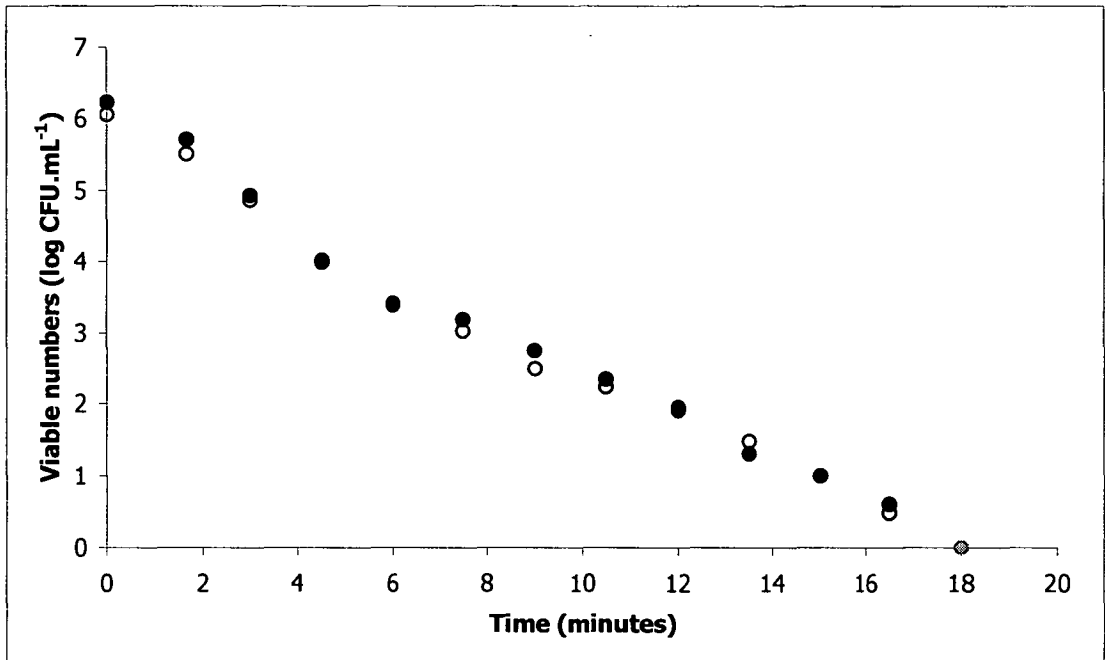
Hazan *et al.* (2004) have reported that the *mazEF* module mediates the death of specific strains of *E. coli* in response to treatment with high temperature. Initial investigations as part of the present work attempted to confirm these findings using a different strain of *E. coli* (MG1655) when some methodologies were modified. Because the *mazEF* system can be non-operational in stationary phase populations of *E. coli* (Hazan *et al.*, 2004) investigations presented in the current chapter used exponential phase cells. Populations of *E. coli* (i.e. the wild-type MG1655 and its  $\Delta mazF$  derivative SC30) were exposed to six elevated temperatures in TSB for 10.0 minutes. The percent fraction survival in each population was calculated as described in Section 5.2.3.3. The average percent survival at each temperature observed in three separate experiments is shown in Figure 5.5. Some of the variability within each data set is attributable to slight variations in the temperature maintained in separate experiments. Results indicate that there was no significant difference ( $P > 0.01$ ) in the percent survival of *E. coli* MG1655 and the  $\Delta mazF$  mutant *E. coli* SC30 at any of the six temperatures tested.



**Figure 5.5** The percent survival of exponential phase populations of *E. coli* (● MG1655, wild-type; ○ SC30,  $\Delta mazF$ ) following exposure to high temperatures in TSB for 10.0 minutes. Viable cells were enumerated on BHA-P at 37°C for 14 hours. The percent survival was determined by comparing the absolute number of viable cells (CFU.mL<sup>-1</sup>) of treated cells to that immediately prior to heat treatment and is shown on a logarithmic scale. Standard deviation ( $n = 3$ ) is shown.

Because the above analysis evaluated the level of inactivation of *E. coli* at a single time point, *mazEF*-mediated cell death at a later time in the inactivation of *E. coli* MG1655 in response to high temperatures could not be discounted. To determine more conclusively if *mazEF* is involved in the heat-induced inactivation *E. coli* MG1655, survival curves were generated for a single temperature [53°C, TSB]. The results of a single experiment, that is representative of another experiment, are shown in Figure 5.6. The viability of each population was similar at all times. Any variation was within the measurement error of the culture-based methodology used (approximately  $\pm 0.3 \log \text{CFU.mL}^{-1}$ ) and so these results indicate that *mazEF* does not mediate the inactivation of *E. coli* MG1655 at 53°C.



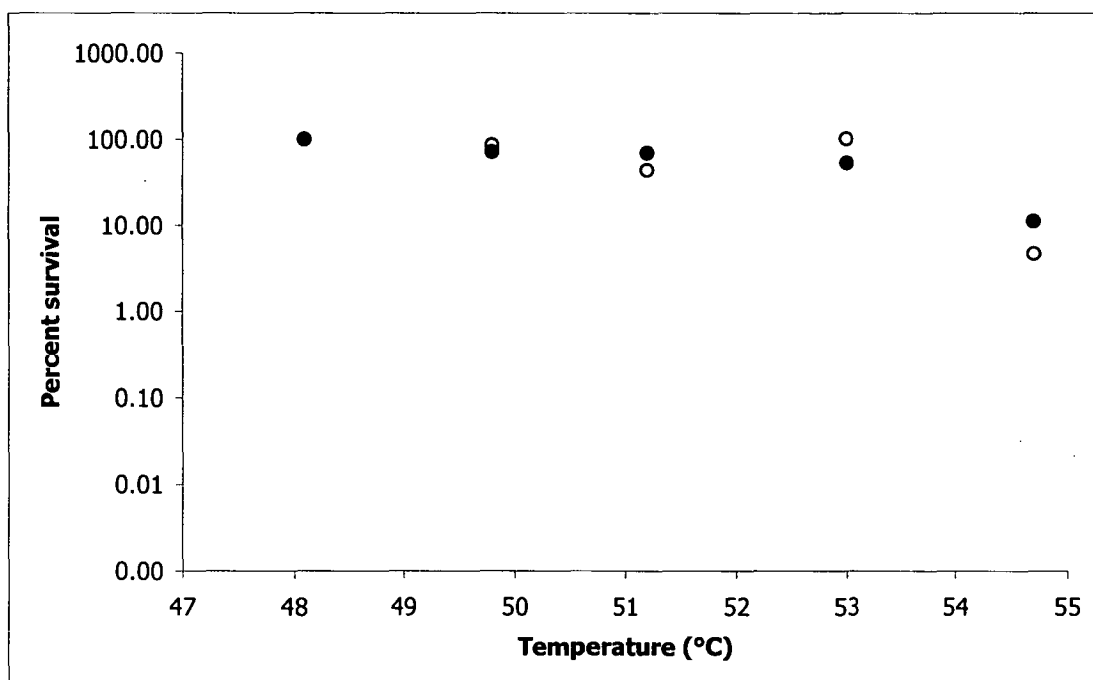


**Figure 5.6** The inactivation kinetics of exponential phase populations of *E. coli* (● MG1655, wild-type; ○ SC30,  $\Delta mazF$ ) at 53°C in TSB. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

The findings of the current work (i.e. that *mazEF* does not mediate death of *E. coli* in response to high temperatures) are in direct contrast to that reported by Hazan *et al.* (2004). Differences in the methodologies used in the two investigations might account for the discrepancies in the results. Firstly, Hazan *et al.* (2004) used the wild-types *E. coli* MC4100 *relA*<sup>+</sup> and *E. coli* K38 and their  $\Delta mazEF$  derivatives (*E. coli* MC4100 *relA*<sup>+</sup> and K38 *mazEF::kan*), whereas the present work used *E. coli* MG1655. Further, the mutant used as part of this study (*E. coli* SC30) contained a disruption to *mazF*, rather than both *mazE* and *mazF*. Based on the current understanding of the *mazEF* system, the single gene deletion of *mazF* as opposed to disruption of the entire *mazEF* module is anticipated to have no influence on the survival characteristics of the strain. Additionally, Christensen *et al.* (2003) have reported that  $\Delta mazEF$  derivatives of *E. coli* MG1655 exhibit a partial *relA* phenotype and so those authors prefer to use the  $\Delta mazF$  *E. coli* mutant.

The method of Hazan *et al.* (2004) further involved transferring the inocula from 37°C to the required high temperature (42 – 52°C) and incubating statically for 10 minutes before the enumeration of viable numbers. By that method, the high temperature incubation was effectively less than 10 minutes because the temperature of the broth had to increase from 37°C in that time. Also, because the broth was incubated statically, the high temperature treatment was probably not applied uniformly throughout the population. To more precisely apply the heat treatment, the inocula used in the present study were diluted to temperature-equilibrated broth in a water bath with shaking.

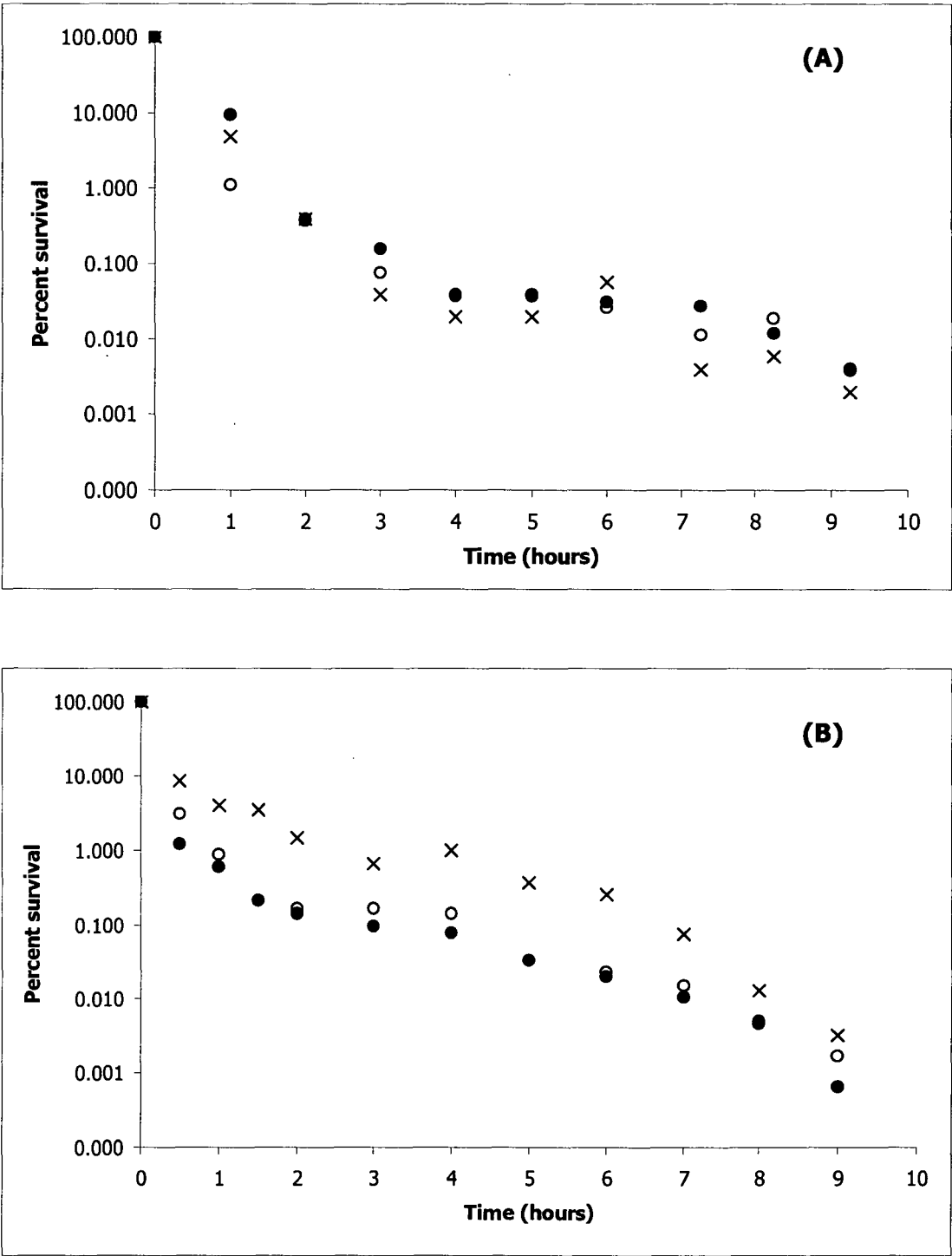
Perhaps the more notable differences between the methodologies of Hazan *et al.* (2004) and that employed during initial studies described here are the preparation of the exponential phase inocula and the suspension media used. The preparation of the inocula in the current work involved dilution of the culture to low numbers in fresh broth to remove residual cells in stationary phase (Brown, 2002). Further, the inocula were prepared, and the populations treated with high temperature in complex broth (TSB) as opposed to minimally defined medium (see Section 2.4.1.2 for a discussion as to the potential influence of the suspension medium on bacterial inactivation). To ascertain whether these variations could account for the inconsistent findings, a single experiment was conducted that more accurately mimicked these aspects of the methodology of Hazan *et al.* (2004). The percent survival in populations of *E. coli* MG1655 and SC30 following treatment with high temperature in this experiment is depicted in Figure 5.7. These results indicate that there is no consistent difference between the inactivation of the two *E. coli* populations and suggests that the methodological aspects adjusted as part of this experiment could not account for the discrepancies between the current work and that reported by Hazan *et al.* (2004).



**Figure 5.7** The log fraction survival of partially-exponential populations of *E. coli* strains MG1655 (●; wild-type) and SC30 (○;  $\Delta mazF$ ) following exposure to high temperatures in MM for 10.0 minutes. Viable cells were enumerated on BHA-P at 37°C for 14 hours and the percent survival was calculated by comparing the absolute number of viable cells (CFU.mL<sup>-1</sup>) following the heat treatment with that immediately prior to the heat treatment and is shown on a logarithmic scale.

### 5.3.2.2 Low Water Activity

This investigation used *E. coli* MG1655 (wild-type) and two of its  $\Delta mazF$  mutants (*E. coli* SC30 and SM01). Each strain was grown to exponential phase and transferred to TSB with low  $a_w$  [ $a_w$  0.95 or 0.90, 25°C, TSB]. The number of viable cells was determined at numerous time points up to 9.5 hours of treatment and the percent survival was determined by comparing the absolute number of viable cells at each sampling point to that immediately prior to the low  $a_w$  treatment. The results of single experiments are shown in Figure 5.8.



**Figure 5.8** The percent survival of exponential phase populations of *E. coli* (● MG1655, wild-type; ○ SC30,  $\Delta mazF$ , and × SM01,  $\Delta mazF$ ) during exposure to (A)  $a_w$  0.95 or (B)  $a_w$  0.90 (NaCl as humectant) at 25°C in TSB. Viable cells were enumerated on BHA-P at 37°C for 14 hours. The percent survival was determined by comparing the absolute number of viable cells (CFU.mL<sup>-1</sup>) of treated cells to that immediately prior to the low  $a_w$  treatment and is shown on a logarithmic scale.

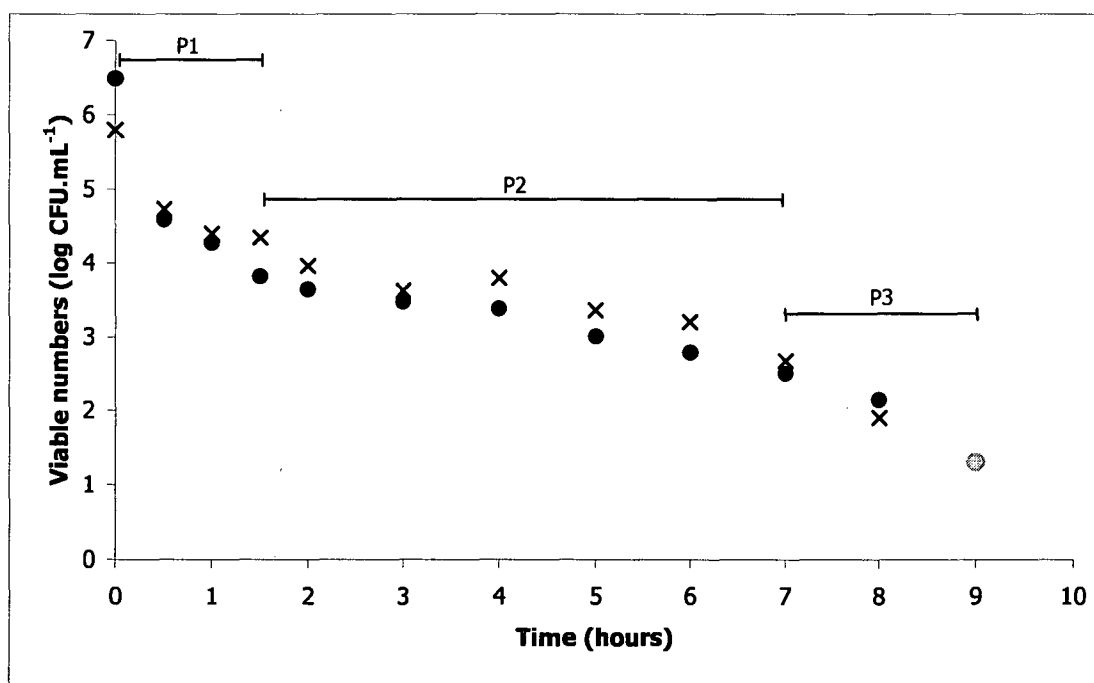
The results depicted in Figure 5.8(A) show that the percent survival of *E. coli* MG1655 and either of its  $\Delta mazF$  derivatives in response to  $a_w$  0.95 is similar. Any variation observed between the three populations is within the measurement error associated with culture-based enumeration (approximately  $\pm 0.3 \log \text{CFU.mL}^{-1}$ ). From this experiment it appears that *mazEF* does not mediate the death of *E. coli* MG1655 in response to  $a_w$  0.95 under the experimental conditions tested.

At  $a_w$  0.90 (see Figure 5.8B), the percent survival of *E. coli* MG1655 is, again, very similar to that of *E. coli* SC30. This was confirmed in two separate experiments. It appears, therefore, that based on a comparison of the percent survival of *E. coli* MG1655 and SC30, that *mazEF* does not mediate the death of *E. coli* in response to  $a_w$  0.90. However, the percent survival of *E. coli* MG1655 does differ from that of *E. coli* SM01 and to an extent that cannot be accounted for by measurement error. This experiment was repeated twice and in all cases, *E. coli* SM01 showed better survival than *E. coli* MG1655. From this comparison, *mazEF* does appear to mediate the death of a proportion of *E. coli* MG1655 following exposure of these cells to  $a_w$  0.90. The use of two  $\Delta mazF$  *E. coli* mutants has highlighted some uncertainties in the involvement of *mazEF* in the low  $a_w$  (0.90)-induced inactivation of *E. coli* MG1655.

With the ambiguity in the above findings recognised, analyses continued to describe the potential functioning of *mazEF* in the low  $a_w$  (0.90)-induced inactivation of *E. coli*, based on a comparison of the survival of *E. coli* MG1655 with *E. coli* SM01. From Figure 5.8(B), it is apparent that *mazEF*-mediated the inactivation of *E. coli* MG1655 within 30 minutes of treatment with low  $a_w$ . After this time, the rate of decline in percent survival of *E. coli* MG1655 and SM01 was similar; indicating that some other process mediates the death of *E. coli* MG1655 at this later stage.

### 5.3.2.3 Characterising the Effect of *mazEF*-Mediated Cell Death on the Kinetics of Low Water Activity-Induced Inactivation

Further to characterising the timing of *mazEF*-mediated cell death, analyses attempted to determine the effect of that *mazEF* involvement on the subsequent inactivation of surviving cells, since it has been suggested that *mazEF*-mediated killing aids the surviving proportion of the population, perhaps by reducing the competition for resources (Aizenman *et al.*, 1996). The inactivation kinetics of exponential phase populations of *E. coli* MG1655 and SM01 exposed to low  $a_w$  [ $a_w$  0.90, 25°C, TSB] are depicted in Figure 5.9, from which the percent survival data given in Figure 5.8(B) were derived.



**Figure 5.9** The inactivation kinetics of exponential phase populations of *E. coli* (● MG1655, wild-type, and × SM01,  $\Delta mazF$ ) in response to  $a_w$  0.90 (NaCl as humectant) at 25°C in TSB. Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours. The horizontal bars indicate the duration of Phases 1, 2, and 3 (P1, P2 and P3, respectively).

The inactivation of *E. coli* MG1655 initially occurred at a rapid rate. After approximately 1.5 hours, the rate of inactivation slowed until around 7 hours and this was followed by a more rapid inactivation rate that continued until viable cells were below the minimum level of detection (i.e. undetected when plating a 50  $\mu\text{L}$  volume to BHA-P and therefore plotted as  $1.3 \log \text{CFU.mL}^{-1}$ ) after 8 hours. Therefore, three phases of inactivation (P1, P2 and P3) were apparent during the inactivation of exponential phase populations of *E. coli* MG1655 in response to  $a_w$  0.90. The inactivation of *E. coli* SM01 similarly showed three phases of inactivation. Characteristics used to describe the kinetics of inactivation (duration, rate of inactivation and loss of viability) were estimated from the survival curves depicted in Figure 5.9 and are given in Table 5.4.

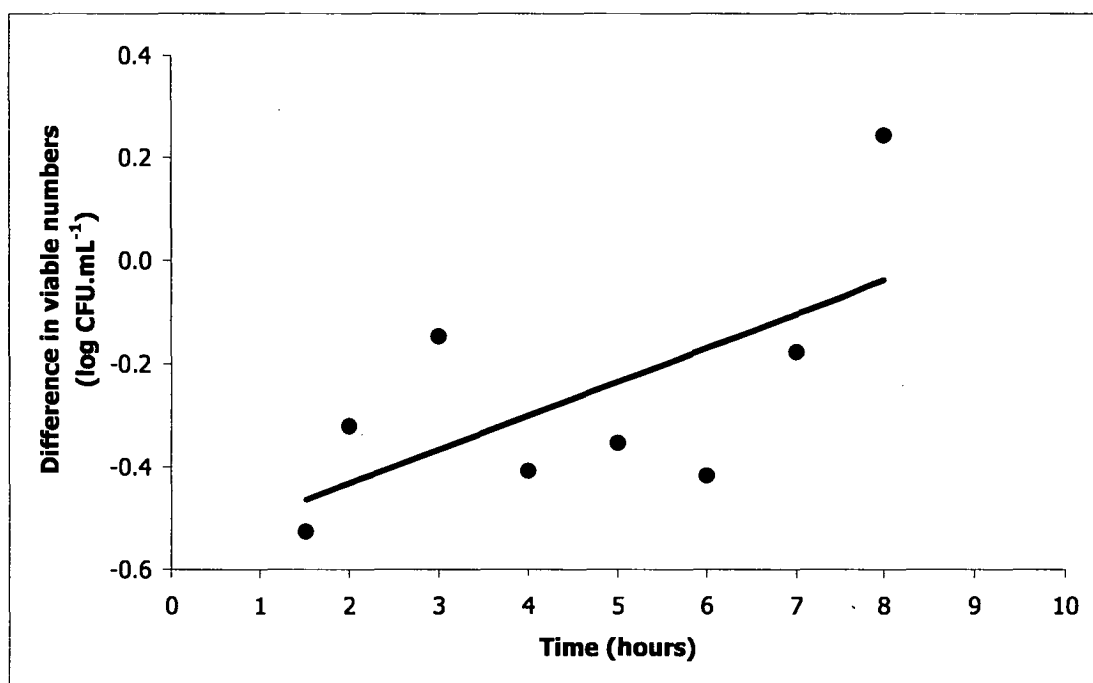
**Table 5.4** The duration, rate of inactivation and loss of viability associated with Phases 1, 2 and 3 in exponential populations of wild-type *E. coli* (MG1655) and  $\Delta mazF$  *E. coli* (SM01) exposed to  $a_w$  0.90. Values were derived from the viability data given in Figure 5.9. The change in the rate of inactivation and loss of viability between strains is shown for each phase.

| <b>Phase of inactivation</b> | <b><i>E. coli</i> strain</b> | <b>Duration (hours)</b> | <b>Rate of inactivation (<math>\log \text{CFU.mL}^{-1}.\text{hr}^{-1}</math>)</b> | <b>Loss of viability (<math>\log \text{CFU.mL}^{-1}</math>)</b> |
|------------------------------|------------------------------|-------------------------|---|---|
| 1                            | MG1655 <sup>a</sup>          | 1.5                     | 1.66  | 2.67  |
|                              | SM01 <sup>b</sup>            | 1.5                     | 0.94  | 1.45  |
|                              | Change <sup>(= a - b)</sup>  |                         | 0.83  | 1.22  |
| 2                            | MG1655 <sup>a</sup>          | 5.5                     | 0.23  | 1.31  |
|                              | SM01 <sup>b</sup>            | 5.5                     | 0.25  | 1.67  |
|                              | Change <sup>(= a - b)</sup>  |                         | -0.02   | -0.36   |
| 3                            | MG1655 <sup>a</sup>          | 2                       | 0.60  | 1.21  |
|                              | SM01 <sup>b</sup>            | 2                       | 0.69  | 1.38  |
|                              | Change <sup>(= a - b)</sup>  |                         | -0.09   | -0.17   |

The results given in Table 5.4 reiterate that *mazEF*-mediated cell death occurs during P1, because the loss in viability during this phase is much greater in *E. coli* MG1655 (with functioning *mazEF*) than in *E. coli* SM01 ( $\Delta mazF$ ). In contrast, the characteristics of P2 and P3 for both strains are very similar, indicating that (a) *mazEF*-mediated inactivation of *E. coli* MG1655 did not occur during the second and third phases of decline in response to  $a_w$  0.90 and (b) *mazEF*-mediated death of a proportion of the *E. coli* MG1655 population during P1 made little difference on the ability of the surviving cells to remain viable in the low  $a_w$  environment.

The latter finding was tested more fully by calculating the difference in viable numbers in the *E. coli* MG1655 and SM01 populations at each sampling point during P2 and P3 (except for the enumeration point at 9 hours, when viable numbers were below the minimum level of detection). These values are given in Figure 5.10. Qualitatively, the viability of *E. coli* MG1655 relative to *E. coli* SM01 increased with time, indicating that the loss of a proportion of the wild-type population by *mazEF* during P1 improved the capacity of surviving cells to maintain functioning under inimical  $a_w$  constraints. However, this systematic variation was not statistically significant ( $r^2 = 0.4148$ ) and, thus, the loss of a proportion of the population mediated during P1 by *mazEF* does not significantly improve the survival characteristics of the remaining population.





**Figure 5.10** The difference in viability in exponential phase populations of *E. coli* MG1655 and SM01 during the second and third phases of inactivation in response to  $a_w$  0.90 (NaCl as humectant) at 25°C in TSB. Values are the number of viable cells in *E. coli* SM01 subtracted from that in *E. coli* MG1655 and were derived from the viability data given in Figure 5.9. The line of best fit to the data is shown ( $r^2 = 0.4148$ ).

## 5.4 DISCUSSION

Osmotically stressed populations of *E. coli* have been shown to minimise the loss of water molecules from the cell by accumulating compatible solutes (Csonka, 1989). At low  $a_w$  levels ( $< 0.95$ ) this response is not adequate to ensure the continued growth of the population and cells either remain dormant or are inactivated. The kinetics of low  $a_w$ -induced inactivation of *E. coli* were described in Chapter 3 of this thesis. Despite the useful application of these quantitative descriptions of bacterial inactivation within the food industry, the actual mechanism(s) involved in osmotic-induced cell death await elucidation. The current work was undertaken to

determine if the inactivation of *E. coli* in response to low  $a_w$  is due to a self-mediated process of programmed cell death.

Specifically, a chromosomally-encoded TA system in *E. coli* (i.e. *mazEF*) was evaluated for its involvement in low  $a_w$ -induced inactivation. Due to time limitations, possible involvement of other TA modules identified in *E. coli* was not ascertained in the current work and so the findings presented here are restricted to the *mazEF* module. Christensen *et al.* (2003) have shown that separate TA modules can mediate death of *E. coli* in response to different triggers and so results of the current study cannot, and should not, be used to predict the involvement of other TA modules.

The construction *E. coli* SM01 formed the initial stage of this work. Although an identical mutant (*E. coli* SC30) was provided by Ms. Susanne Christensen (Christensen *et al.*, 2003), the construction of *E. coli* SM01 allowed for the development of a standardised method to produce essentially any *E. coli* mutant within our laboratories at the University of Tasmania and, therefore, was of considerable value. In relation to the survival studies described in this chapter, *E. coli* SM01 was only employed in later experiments (i.e. those that focussed on osmotically stressed *E. coli* populations) because all other trials had been completed (using *E. coli* SC30) at the time of its construction. It should be noted that both of the mutants employed were derived from *E. coli* MG1655. While investigations presented in previous chapters of this thesis have used relatively stress-resistant stationary phase populations of *E. coli* M23, the functioning of *mazEF* in that strain has not been reported. Further, the *mazEF* system can be non-operational in stationary phase populations of *E. coli* (Hazan *et al.*, 2004) and so survival studies described in this chapter used exponentially growing inocula of *E. coli* MG1655.

Preliminary investigations as part of the present study attempted to confirm the findings of Hazan *et al.* (2004), that *mazEF* mediates death of *E. coli* in response to high temperature treatments. This was intended to determine the potential of *mazEF* to function in *E. coli* suspended in complex broth, since the study of PCD in bacteria has tended to use minimal media (Sat *et al.*, 2001; Christensen *et al.*, 2003; Sat *et al.*, 2003; Christensen *et al.*, 2004; Hazan *et al.*, 2004), which could potentially complicate comparisons with data presented throughout this thesis that use TSB. Initial results indicated that there was no significant difference between the survival of *E. coli* SC30 ( $\Delta mazF$ ) and its wild-type (*E. coli* MG1655) at 49.9, 51.0, 52.2, 52.8, 54.0 and 55.1°C following 10 minutes of incubation in TSB. Further, the *mazEF* system did not mediate the death of *E. coli* MG1655 at a later stage in the inactivation of the population at high temperature (53°C), as determined by assessment of bacterial viability at regular intervals over an extended time period. Even when the methods of Hazan *et al.* (2004) were carefully followed, and used MM rather than TSB, involvement of *mazEF* in the high temperature-induced inactivation of *E. coli* MG1655 was not apparent. These findings collectively indicate that *mazEF* may not mediate the death of *E. coli* MG1655 due to high temperature treatments under the various conditions tested.

The series of high temperature experiments described in this chapter and the results of Hazan *et al.* (2004) collectively suggest that the involvement of *mazEF* in the inactivation of *E. coli* in response to high temperatures is strain specific or that the *mazEF* system operates only under a very specific set of conditions. Importantly, the results presented by Hazan *et al.* (2004) appeared in the Notes section of the Journal of Bacteriology and were, therefore, intended as a presentation of brief observations. The results were not statistically evaluated and showed the percent survival of the relevant *E. coli* populations determined in a

single experiment that were said to be representative of three independent trials. At the time of writing of this thesis, *mazEF*-mediated cell death in response to high temperatures had not been further reported in the literature. In any case, the functioning of *mazEF* (i.e. a proteic cell death-mediating system) in the high temperature-induced inactivation of *E. coli* would appear contradictory, given that the mechanism of high temperature-induced inactivation of bacterial cells is usually attributed to denaturation of cellular proteins (Gould, 1989a). Clearly, the potential involvement of TA systems as mediators of high temperature-induced inactivation of bacterial populations awaits better elucidation.

The *mazEF* system has been reported to mediate the death of *E. coli* following exposure to non-thermal stresses, such as UV irradiation (Hazan *et al.*, 2004), amino acid and thymine starvation (Engelberg-Kulka *et al.*, 1998; Sat *et al.*, 2003), and treatment with some antibiotics (Sat *et al.*, 2001). It was postulated that *mazEF* might also be involved in the decline of *E. coli* populations following exposure to other, as yet untested, inimical processes. Despite the above findings, that suggest the *mazEF* system does not operate in *E. coli* MG1655 when this microorganism is suspended in complex broth, investigations were continued to determine if this TA module mediates the low  $a_w$ -induced inactivation of *E. coli* MG1655 in TSB. Results suggested that *mazEF* does mediate the death of *E. coli* MG1655 in populations exposed to low  $a_w$ , however, this involvement is in some way dependent on the level of stress imposed. At  $a_w$  0.90 the  $\Delta mazF$  *E. coli* mutant SM01 showed better survival than the wild-type strain, indicating that, when encoded on the chromosome, *mazEF* mediates the death of *E. coli* in response to that  $a_w$  stress. In contrast, when the  $a_w$  of the medium was 0.95, no consistent difference in the survival of these strains was observed. Hazan *et al.* (2004) have reported that *mazEF* mediates the death of *E. coli* in response to a limited range of

stressful conditions and this study has reiterated that, by showing that the involvement of *mazEF* in low  $a_w$ -induced inactivation of *E. coli* depends on the severity of the applied osmotic stress.

The potential involvement of the *mazEF* module in the inactivation of *E. coli* in response to low  $a_w$  (0.90) conditions is somewhat undermined by the finding that the survival characteristics of a second  $\Delta mazF$  *E. coli* mutant (SC30) were not consistently altered from that of the wild-type. That the  $a_w$  0.90-induced inactivation of the two  $\Delta mazF$  *E. coli* mutants differed considerably is somewhat puzzling. Both mutants were constructed using the Red helper system (Datsenko and Wanner, 2000), the same parental strain and identical primers, and nucleotide sequence analyses of the mutants indicated that they were structurally identical. Therefore, *E. coli* SC30 and SM01 differed only in their culture history prior to storage of stock populations at  $-80^\circ\text{C}$  and while this could affect their response to osmotic stress it is unlikely to do so to such a large extent.

Further investigation is required to resolve the discrepancies apparent between *E. coli* SC30 and SM01 and thereby alleviate the ambiguities associated with *mazEF*-mediated death in response to low  $a_w$  conditions. In the very least this should involve constructing, or otherwise obtaining, a third  $\Delta mazF$  derivative of *E. coli* MG1655 and testing its survival characteristics. Otherwise, experiments that do not rely upon the use of  $\Delta mazF$  mutants could be initiated. One viable approach is to measure the level of MazE expression in *E. coli* during treatment with low  $a_w$  via a series of western blot analyses. A reduction in MazE expression would indicate that MazF is free to mediate cell death. Until the inconsistencies in behaviour of the  $\Delta mazF$  *E. coli* mutants are accounted for, or *mazEF*-mediated cell death is confirmed using some other methodology, the involvement of *mazEF* in the death of *E. coli* in

response to a limited set of conditions reported in this work is considered ambiguous. The possibility of *mazEF*-mediated cell death is discussed further below while acknowledging the uncertainties within the data.

Where the *mazEF* TA system appeared to mediate the death of a proportion of the *E. coli* MG1655 population exposed to low  $a_w$  conditions (i.e.  $a_w$  0.90, based on a comparison of the wild-type and the  $\Delta mazF$  *E. coli* mutant SM01), this occurred within 30 minutes of exposure of *E. coli* MG1655 to low  $a_w$  and, therefore, during the first (P1) of three phases of inactivation. After that time, the *E. coli* populations declined similarly suggesting that the *mazEF* system was not involved in the death of *E. coli* MG1655 during the later stages of inactivation (P2 and P3). Although this is yet to be confirmed in *stationary* phase populations of *E. coli* exposed to lethal  $a_w$ , the current work suggests that the increased rate of inactivation observed during P3 (described in Section 3.4.1) is not due to a *mazEF*-mediated process.

To the best of the candidate's knowledge, this study is the first to describe the occurrence of *mazEF*-mediated death at a *distinct* stage during the inactivation of *E. coli* populations. The majority of publications reporting on the involvement of *mazEF* in response to stressful environments have been authored by members of Hanna Engelberg-Kulka's research group at the Hebrew University-Hadassah Medical School in Israel (Engelberg-Kulka *et al.*, 1998; Sat *et al.*, 2001; Sat *et al.*, 2003; Hazan *et al.*, 2004). Those researchers have focussed on the initial stage of bacterial inactivation in response to stress (i.e. when there is less than 1-log kill, or at least 10% survival), as suggested by their presentation of percent survival data that does not use a logarithmic scale. In this way, those authors highlight the difference in survival between the wild-type and its  $\Delta mazEF$  derivative before 1-log kill is achieved but obscure any differences that may occur following further

inactivation. The results of those authors support the present finding that *mazEF*-mediated cell death occurs at the initial stages of stress application. However, the level of *mazEF*-mediated cell death in later stages in the population's decline cannot be ascertained from those publications and, thus, it is unclear whether *mazEF*-mediated cell death is a feature exclusive to the initial stages of inactivation, as shown in populations of *E. coli* MG1655 exposed to  $a_w$  0.90 in this work.

It is a widely accepted hypothesis that bacterial cells respond to inimical conditions to counteract detrimental effects so that cellular functioning can continue. Although the programmatic elimination of *E. coli* in response to low  $a_w$  appears contradictory to such processes that essentially promote bacterial survival, it is possible that the early-onset of *mazEF*-mediated cell death benefits the community as a whole (Shapiro, 1998), perhaps by providing nutrients to surrounding cells and decreasing the competition for resources (Aizenman *et al.*, 1996). However, analyses of the effect of *mazEF*-mediated cell death (that occurred during P1) on the subsequent survival characteristics of *E. coli* (during P2 and P3) conducted in this work indicated that *mazEF* involvement did not significantly alter the survival characteristics of *E. coli* MG1655. As a result, the functional reason for *mazEF*-mediated cell death in *E. coli* exposed to low  $a_w$  remains to be experimentally shown. Given that the concept of PCD in bacterial cells is an emerging one, it is likely that its functional role will not be fully elucidated until knowledge pertaining to the involvement of PCD in many different systems is characterised.

Based on the results presented in this chapter, and concepts that have arisen throughout this thesis, a number of avenues are proposed for future research relating to PCD in *E. coli*. Firstly, the involvement of other TA modules (e.g. *chpB* and *relBE*) in the low  $a_w$ -induced inactivation of *E. coli* should be determined using

the appropriate *E. coli* mutants and the methodologies described in the current work. Hazan *et al.* (2001) have suggested that some TA modules interact to mediate PCD and so the use of multiple gene deletion mutants would be invaluable in more comprehensively describing the processes of PCD in low- $a_w$  induced inactivation of *E. coli*. The characterisation of *mazEF*-mediated cell death in *E. coli* in response to other stresses (e.g. low pH, treatment with organic acids, etc.) would be beneficial in improving the understanding of the inactivation processes related to those specific stresses as well as providing an indication of the sphere of influence of PCD in bacterial inactivation. A comprehensive description of the specific conditions that induce a self-mediated process of cell death in bacteria has the potential to be exploited within the food industry to more effectively reduce the viability of pathogens in foods and, thereby, reduce the risk of foodborne illness.

The present work has demonstrated that the *mazEF* system mediates the death of only a proportion of the *E. coli* MG1655 population in response to low  $a_w$  conditions. Therefore, the destruction of other cells within the population must be due to some other mechanism. While the death of a cell is generally assumed to occur because the cell is unable to function properly, due to damage to enzyme and protein synthesis pathways (Sykes, 1963), the exact cellular components that mediate this event have not been experimentally proven. Further, even non-lethal damage to different cellular components (e.g. DNA and RNA molecules) in *E. coli* exposed to low  $a_w$  remains to be definitively shown. Damage to these components might infer a lethal effect (i.e. if further exposure to low  $a_w$  causes more damage to that component). Membrane-associated injury has already been recognised as a potential site of damage in *E. coli* M23 due to low  $a_w$  (Chapter 3). Future experiments might identify other sites of damage by using repair-deficient strains of *E. coli* and comparing the low  $a_w$ -induced lethality in such populations with the



appropriate wild-type strain. For example, should low  $a_w$  cause enhanced inactivation of *E. coli* when that population is deficient in DNA polymerase I (i.e. due to deletion of the *polA1* gene), that would indicate that osmotic stress causes DNA damage that requires DNA polymerase I for repair (Sinha, 1986). A series of similar investigations would provide information relating to the specific cellular components that are damaged by low  $a_w$  and would highlight the processes involved during the decline of bacterial populations.

#### **5.4.1 Conclusions**

The identification of a number of distinct genetic elements (i.e. TA modules) that are able to mediate the death of bacterial cells in response to environmental stresses constitutes an interesting development in the current understanding of bacterial inactivation. Although the study of bacteria in inimical environments often focuses on the potential for microorganisms to survive and the specific responses that allow them to do so, knowledge of the mechanisms that actually kill some, or all of the cells in that population is also of particular interest. A series of experiments pertaining to the inactivation of *E. coli* MG1655 in response to high temperatures and low  $a_w$  were presented in this chapter. Under all of the conditions tested, the TA system *mazEF* did not appear to mediate the heat-induced inactivation of *E. coli* MG1655. Further experiments indicated that *mazEF* did not mediate *E. coli* MG1655 inactivation in response to  $a_w$  0.95 but the same system may have functioned in *E. coli* MG1655 exposed to a more intense  $a_w$  stress (i.e.  $a_w$  0.90). The latter finding was complicated by variation in the survival characteristics of two  $\Delta mazF$  *E. coli* mutants that, theoretically, should have exhibited identical behaviour. As a result, the mechanism of cell death in *E. coli* MG1655 exposed to  $a_w$  0.90 could not be conclusively shown to involve the *mazEF* system but nor could

its association be ruled out. Where *mazEF* did appear to mediate the death of *E. coli* MG1655 exposed to an osmotic stress that occurred during the initial stages of the inactivation profile. That early-onset, *mazEF*-mediated death of a proportion of the population did not improve the survival characteristics of the remaining cells and so the reason for the functioning of *mazEF* in low  $a_w$ -induced inactivation of *E. coli* remains to be experimentally demonstrated. Further investigations to characterise the participation of these genetically controlled, self-mediated bacterial cell death processes in response to osmotic stress and other inimical environments has the potential to greatly enhance the current knowledge of bacterial death and survival and may have practical applications within the food industry. However, TA systems are unlikely to account for the inactivation of the entire population in response to low  $a_w$  treatments and so other mechanisms of inactivation need to be considered.

**Chapter 6**

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**Investigating Specific Responses  
to Lethal Water Activity Stress**

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## 6.1 INTRODUCTION

The lethal effect of low  $a_w$  on pathogenic bacteria has been widely exploited in food manufacturing processes to reduce the number of microorganisms present. At  $a_w$  values relevant to a number of food products, however, the inactivation of *E. coli* is not instantaneous and some cells are able to survive for relatively extended periods. Assessment of the viability of *E. coli* populations exposed to  $a_w$  0.90 (Chapter 3) clearly demonstrated the existence of subpopulations of *E. coli* better able to survive that stress. It is those subpopulations of cells that are of particular concern to food manufacturers, especially because pathogenic *E. coli* can cause disease when present in low numbers (Tilden *et al.*, 1996). Despite their importance, and a general view that knowledge of bacterial interactions with their environment can improve food processing practices, the physiology of cells that survive in lethal  $a_w$  environments is poorly understood.

Of foremost interest is whether cells that survive 'lethal'  $a_w$  stress do so by initiating an active (i.e. energy-utilising) and/or stress-specific response, or whether they are inherently more resistant to the stress, for whatever reason, and their survival is essentially a passive process. Studies that have focussed on 'mild'  $a_w$  stresses (i.e. growth-limiting but non-lethal) have shown that bacteria minimise the detrimental effect of low  $a_w$  via an active process, termed osmoregulation. The osmoregulatory response was described in Section 1.3 and has been extensively reviewed elsewhere (Csonka, 1989; Gould, 1989b; Galinski and Truper, 1994; Gutierrez *et al.*, 1995; Csonka and Epstein, 1996; Beales, 2004). In *E. coli*, osmoregulation involves the accumulation of compatible solutes via uptake from external sources (e.g. of betaine) and/or synthesis (e.g. of trehalose). The expression of a number of genes is altered during this process, which is manifested as an increase or decrease in the

levels of certain proteins. These genes, and the function of their protein product, are summarised in Table 5.1.

**Table 5.1** Genes, and the function of their protein product, involved in the osmoregulatory response of *E. coli*.

| Gene              | Function  | Change in expression <sup>a</sup> | Reference  |
|-------------------|---|-----------------------------------|--|
| <i>aqpZ</i>       | Aquaporin that allows enhanced transport of water across the cell membrane                  | +                                 | Calamita <i>et al.</i> (1995)                    |
| <i>bet</i>        | Choline uptake  | +                                 | Eshoo (1988)                                     |
| <i>dps</i>        | Non-specific DNA-binding protein that protects DNA against various stresses                 | +                                 | Weber and Jung (2002)                            |
| <i>kdp</i> operon | Potassium uptake system   | +                                 | Laimins <i>et al.</i> (1981)                     |
| <i>ompF</i>       | Outer membrane porin for the passive transport of small solutes                             | -                                 | Csonka (1989)                                    |
| <i>ompC</i>       | Outer membrane porin for the passive transport of small solutes                             | +                                 | Csonka (1989)                                    |
| <i>osmY</i>       | Periplasmic protein, function unknown   | +                                 | Weber and Jung (2002)                            |
| <i>otsA</i>       | Trehalose synthesis   | +                                 | Giaever <i>et al.</i> (1988)                     |
| <i>otsB</i>       | Trehalose synthesis   | +                                 | Giaever <i>et al.</i> (1988)                     |
| <i>proP</i>       | Transport of compatible solutes   | +                                 | Jovanovich <i>et al.</i> (1988)                  |
| <i>proU</i>       | Transport of compatible solutes, not expressed in cells that are not osmotically challenged | +                                 | Gowrishankar (1985), Barron <i>et al.</i> (1986) |
| <i>treA</i>       | Catabolism of trehalose   | +                                 | Gutierrez <i>et al.</i> (1989)                   |

<sup>a</sup> Increase (+)/decrease (-).

The ability of the osmoregulatory response to maintain turgor and essential cellular functioning in *E. coli* exposed to growth limiting but non-lethal  $a_w$  conditions has been firmly established. At external  $a_w$  values of greater than approximately 0.95, *E. coli* populations continue to grow, although at a reduced rate compared to their unstressed counterparts (Troller, 1987). Importantly, this protective effect is

limited. Exposure of *E. coli* to more stringent osmotic stresses results in a loss in viability, presumably because the osmoregulatory capacity of the cell is exceeded and some function essential for survival is inhibited (Beales, 2004). Importantly, the actual mechanisms that mediate the lethal effect of low  $a_w$  in *E. coli* are not known. It is, therefore, difficult to speculate on what responses, if any, *E. coli* might initiate to prevent their inactivation and ensure their survival in lethal  $a_w$  environments. It is possible that the same processes that form part of the osmoregulatory response to mild  $a_w$  stress are initiated when *E. coli* are exposed to the analogous *lethal* constraint. This would imply that the mechanisms that aid cells in their ability to multiply in growth limiting environments also prevent the inactivation of the cell. Also considered to be prime candidate components are proteins identified to be involved in the response of *E. coli* to other inimical constraints, such as heat and acid stress.

The investigation described in this chapter attempted to determine if *E. coli* initiates a stress-specific response to survive potentially lethal osmotic stress. Initial experiments addressed the involvement of the compatible solute betaine in the survival of *E. coli* M23. Subsequently, protein expression in cells that survive lethal  $a_w$  stress for an extended period was assessed using a proteomic approach. The results were analysed for the potential involvement of an osmotically-induced stress response, based primarily on the expression of stress-related proteins.

## **6.2 MATERIALS AND METHODS**

### **6.2.1 Bacterial Strains, Reagents, Media, Solutions and Equipment**

Details of bacterial strains, chemical reagents, bacteriological media, solutions, and equipment (including software and databases) are given in Appendix A. All experiments described in this chapter employed the non-pathogenic *E. coli* M23.

### **6.2.2 Assessment of the Effect of Betaine on the Survival of *Escherichia coli* in Response to Lethal Water Activity**

Stationary phase populations (2.2.2.1) of *E. coli* M23 (2.2.2.2) were exposed to low  $a_w$  [ $a_w$  0.90, 25°C] or low  $a_w$  in combination with low pH [ $a_w$  0.90, pH 3.5, 25°C] in MM in the presence (MM-B) and absence (MM) of 2mM betaine (2.2.2.2). Immediately prior to the treatment, and at regular intervals throughout, the number of viable cells was determined by culture-based methods (2.2.2.4) up until 65 hours.

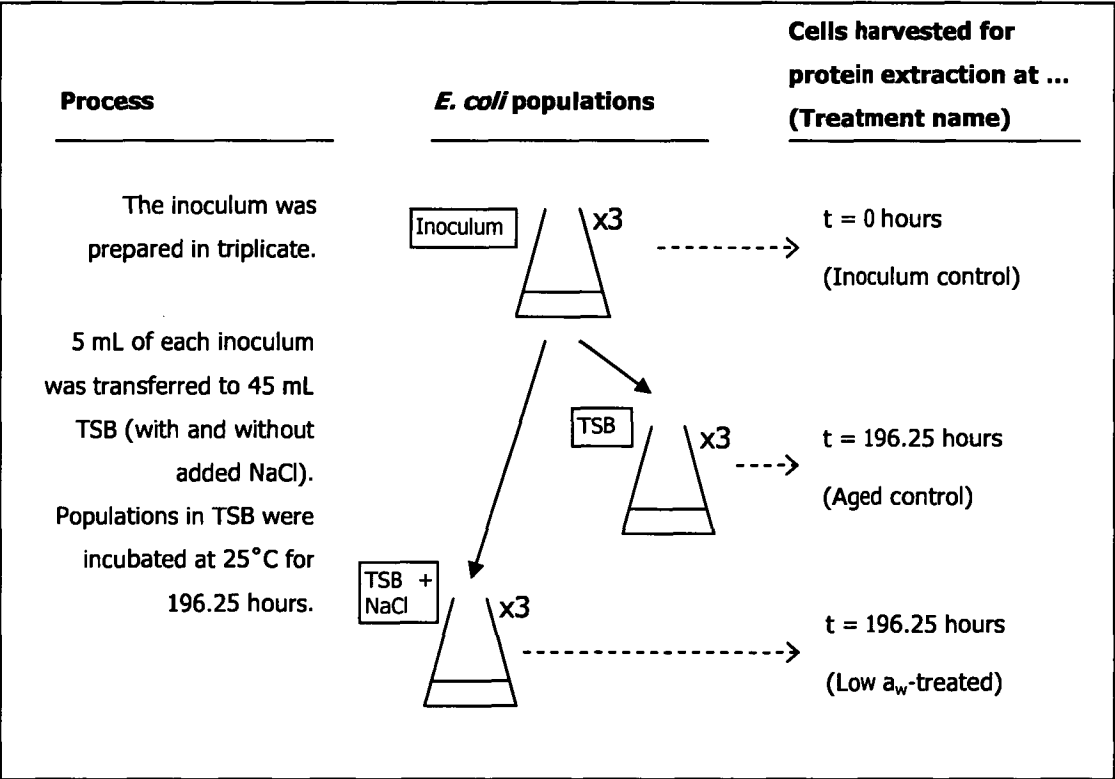
### **6.2.3 Evaluation of Protein Expression in *Escherichia coli* that Survive Lethal Water Activity**

All centrifugation steps were at RT in a Microcentrifuge 5417R unless otherwise stated. Latex gloves were worn during all experiments and during the preparation of solutions to minimise contamination of samples by protease.

#### **6.2.3.1 Low Water Activity Treatment and Harvesting of Cells for Protein Extraction**

Aliquots (5 mL) of stationary phase populations (2.2.2.1) of *E. coli* M23 prepared in triplicate were harvested and inoculated to low  $a_w$  TSB (in triplicate) as described in Section 2.2.2.3 except that the wash step was excluded. A control treatment was

prepared similarly except that no NaCl was added to the TSB. Two control populations (named 'inoculum' and 'aged') and the  $a_w$ -stressed population were harvested for protein extraction, each in triplicate. The inoculum control [no  $a_w$  stress, 25°C, TSB, 0 hours] was harvested immediately following inoculation using the remaining volume of the inoculum (approximately 70 mL). The entire volumes (approximately 48 mL) of both the aged control [no  $a_w$  stress, 25°C, TSB, 196.25 hours] and the  $a_w$ -stressed population [ $a_w$  0.90, 25°C, TSB, 196.25 h] were harvested following 196.25 hours of treatment. The timings and processes involved in this harvest are shown in Figure 6.1.



**Figure 6.1** Diagrammatic representation of the treatments and processes used prior to the extraction of proteins from *E. coli* populations.



During harvesting, cells were kept on ice and centrifuged at 4°C to inhibit the action of bacterial proteases. Specifically, the population was transferred to a sterile Nunc bottle and centrifuged at 10 000 rpm for 1 hour in a J2-21M/E centrifuge. The supernatant was poured-off and the pellet washed twice by suspension in 50 mL low salt buffer in a sterile 50 mL falcon tube and centrifuging at 4300 rpm for 15 minutes in a Sorvall® SUPER T21 centrifuge. The pellet was resuspended in 2 - 3 mL low salt buffer and 1 mL volumes were transferred to pre-weighed sterile microcentrifuge tubes. The suspension was centrifuged at 10 000 rpm for 15 minutes, the mass of the wet pellet determined, and then stored at -20°C.

#### **6.2.3.2 Protein Extraction and Determination of Protein Concentration**

The cell pellet was recovered from -20°C and resuspended in multiple surfactant solution (MSS) to a final concentration of approximately 1 mg cell pellet/10 µL MSS. The suspension was kept on ice and sonicated at 9 Amps for 20 seconds, three times, with a one minute incubation on ice between each sonication. Benzonase (150 units per mL of sonicate) was added and the solution incubated at RT for 20 minutes. Tubes were centrifuged at 14 000 rpm for 15 minutes at 15°C and the supernatant was removed to a sterile microcentrifuge tube and held on ice until the protein concentration of the sample was determined.

The protein concentration was determined using *RC DC*<sup>TM</sup> protein assay reagents according to the manufacturer's instructions. All incubations were at RT. Briefly, the supernatant was diluted (1/10 and 1/100) in sequential extraction reagent 1. Bovine plasma  $\gamma$ -globulin of known concentration (typically 0.2 – 1.5 mg/mL) was diluted (1/5; 1/10; 1/30 and 1/100) in sequential extraction reagent 1 to obtain a protein standard curve. To 25 µL of the diluted sample or standard, 125 µL RC reagent I was added and the solution was incubated for 1 minute. 125 µL RC

reagent II was added and the solution was centrifuged at 14 000 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 127  $\mu$ L DC reagent A supplemented with 2% DC reagent S and incubated for 5 minutes. 1 mL DC reagent B was added and the solution was incubated for a further 15 minutes. The solution was transferred to a microtitre plate and the absorbance was read at 655 nm using a microplate reader and Microplate Manager software. From the protein concentration determined, aliquots (typically 2.5 - 10  $\mu$ L) containing  $\sim$  100  $\mu$ g protein were transferred to sterile microcentrifuge tubes and stored at  $-20^{\circ}\text{C}$ .

### ***6.2.3.3 Isoelectric Focusing (First Dimension)***

Aliquots containing approximately 100  $\mu$ g protein were recovered from  $-20^{\circ}\text{C}$  storage and mixed with 5  $\mu$ L 0.1% bromophenol blue. The volume was adjusted to 190  $\mu$ L with MSS. The entire volume was transferred to a rehydration tray and an 11 cm pH 3 – 10 non-linear ReadyStrip<sup>TM</sup> immobilised pH gradient (IPG) strip was placed over the solution with the gel-side down. The strip was allowed to rehydrate by incubation at RT for 1 hour and was then overlayed with 2 mL mineral oil and incubated at RT overnight (minimum of 15 hours). IPG strips of reduced pH range (pH 4 – 7 linear) were rehydrated as above but the tributylphosphine component of the MSS was replaced with Destreak<sup>TM</sup> reagent (A.1.4) to improve protein solubility and therefore the resolution of proteins in the first dimension. The wire electrodes of an isoelectric focusing (IEF) tray were covered with electrode wicks previously moistened with 8  $\mu$ L sterile Milli-Q  $\text{H}_2\text{O}$ . The rehydrated strips were blotted onto filter paper to remove excess mineral oil and transferred, gel-side down, to the IEF tray. The strip was overlayed with mineral oil, the tray placed in an IEF cell and the proteins were focussed according to their isoelectric point (pI) as follows:

1. Slow ramp to 250 V (1 hour)
2. Slow ramp to 500 V (1 hour)
3. Rapid ramp to 8000 V (20 000 V-hour)
4. Rapid ramp to 500 V (hold).

At completion of the IEF (i.e. at any stage during Step 4), the strip was transferred, gel-side up, to a rehydration tray and stored at  $-20^{\circ}\text{C}$ .

#### ***6.2.3.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (Second Dimension)***

After first dimension focussing, IPG strips were incubated in equilibration base buffer containing 2% dithiothreitol at RT for 10 minutes with gentle agitation (30 rpm) on a platform mixer to reduce sulfhydryl groups on the proteins. The strip was immersed in equilibration base buffer containing 2.5% iodoacetamide and incubated as above to alkylate the sulfhydryl groups and prevent the formation of disulfide bonds. The strip was briefly immersed in 1X tris glycine sodium dodecyl sulphate (TGS) buffer, loaded to a Criterion™ 12.5% tris hydrochlorate resolving gel and overlayed with 1% agarose solution. Protein unstained standards were run on each gel. When the agarose solution had set, the sodium dodecyl sulphate-polyacrylamide gel electrophoresis step was run in an electrophoresis cell (Criterion or Criterion Dodeca) with 1X TGS buffer at 100 V for 15 minutes and then at 200 V until the dye front had migrated off the bottom of the gel.

#### ***6.2.3.5 Detection of Protein***

All incubations were at RT with gentle agitation (30 rpm) on a platform mixer. The gel was incubated in SYPRO Ruby fixative for 1 hour and then stained in SYPRO Ruby protein gel stain overnight (minimum 15 hours). The gel was washed in

SYPRO Ruby fixative for 30 minutes and then in Milli-Q H<sub>2</sub>O for 15 minutes. The gel was visualised using an image scanner and STORMScan software and the image analysed as described below (Section 6.2.3.7). The gel was stored in Milli-Q H<sub>2</sub>O at 4°C until protein spots were excised for identification purposes (2 months maximum) and then in 10 mL gel storage solution in a plastic bag at -20°C.

#### **6.2.3.6 Protein Identification**

Protein spots were excised from gels using a spot cutter and loaded into wells of a microtitre tray containing 50 µL of Milli-Q H<sub>2</sub>O. Samples were digested with trypsin and analysed by MALDI-TOF MS to generate peptide mass fingerprints at Primary Industries and Research Victoria. Milli-Q H<sub>2</sub>O was removed from the wells with a pipette and the gels destained by incubating in 50 µL of 25 mM ammonium bicarbonate (pH 8.0) at RT for 10 minutes. The gels were dehydrated in 50% acetonitrile at RT for 10 minutes. The destaining and dehydration processes were repeated. The samples were dried by incubation at 37°C for 15 minutes and were then incubated at RT for 30 minutes. A 5 µL volume of trypsin solution was added to the sample and incubated at RT for 10 minutes. Excess trypsin was removed and the gel was immersed in 10 µL of 25 mM ammonium bicarbonate. The sample was overlayed with foil and incubated at 37°C for 15 hours. The trypsin-digested samples were ionised by the addition of 1% trifluoroacetic acid (1 µL per 10 µL of peptide digest). Using C18 zip-tips previously wetted with a solution of 50% acetonitrile and equilibrated with a 0.1% trifluoroacetic acid, a 10 µL volume of the peptide solution was pipetted up and down slowly, thus binding the peptides to the tip. The C18 zip-tip was washed with 10 µL of 0.1% trifluoroacetic acid at least five times to remove contaminants. The peptide solution was eluted from the tip to the target using 1.5 µL of extraction solution. The masses of peptides within each

sample were determined using a MALDI-TOF mass spectrophotometer. The resultant peptide mass fingerprints were analysed using Data Explorer software.

The peptide mass fingerprints obtained and the candidate matched those to known proteins by searching of genomic databases using Mascot software. The following parameters were used in the searches: *E. coli* (taxonomy), trypsin digest (one missed cleavage allowed), carbamidomethyl of cysteines (fixed modification), oxidation of methionines (variable modification), peptide mass tolerance of  $\pm 100$  ppm of experimental peptide mass value, and based on monoisotopic and  $MH^+$  peptide mass values. Peptide mass fingerprints were searched against National Center for Biotechnology Information (NCBI) non-redundant database and then against SwissProt database. Only protein matches that returned a significant probability-based molecular weight search (Mowse) score in either database (i.e.  $\geq 57$  and  $\geq 53$  using NCBI and SwissProt respectively) were considered successfully identified (Pappin *et al.*, 1993). The molecular weight and pI values calculated for the matched protein were compared to the location of the protein spot on the two-dimensional electrophoresis (2-DE) gel from which the protein spot was excised to confirm the identification. The function of the identified protein was determined by searching the UniProt protein knowledge database.

#### **6.2.3.7 Comparative Expression Analysis**

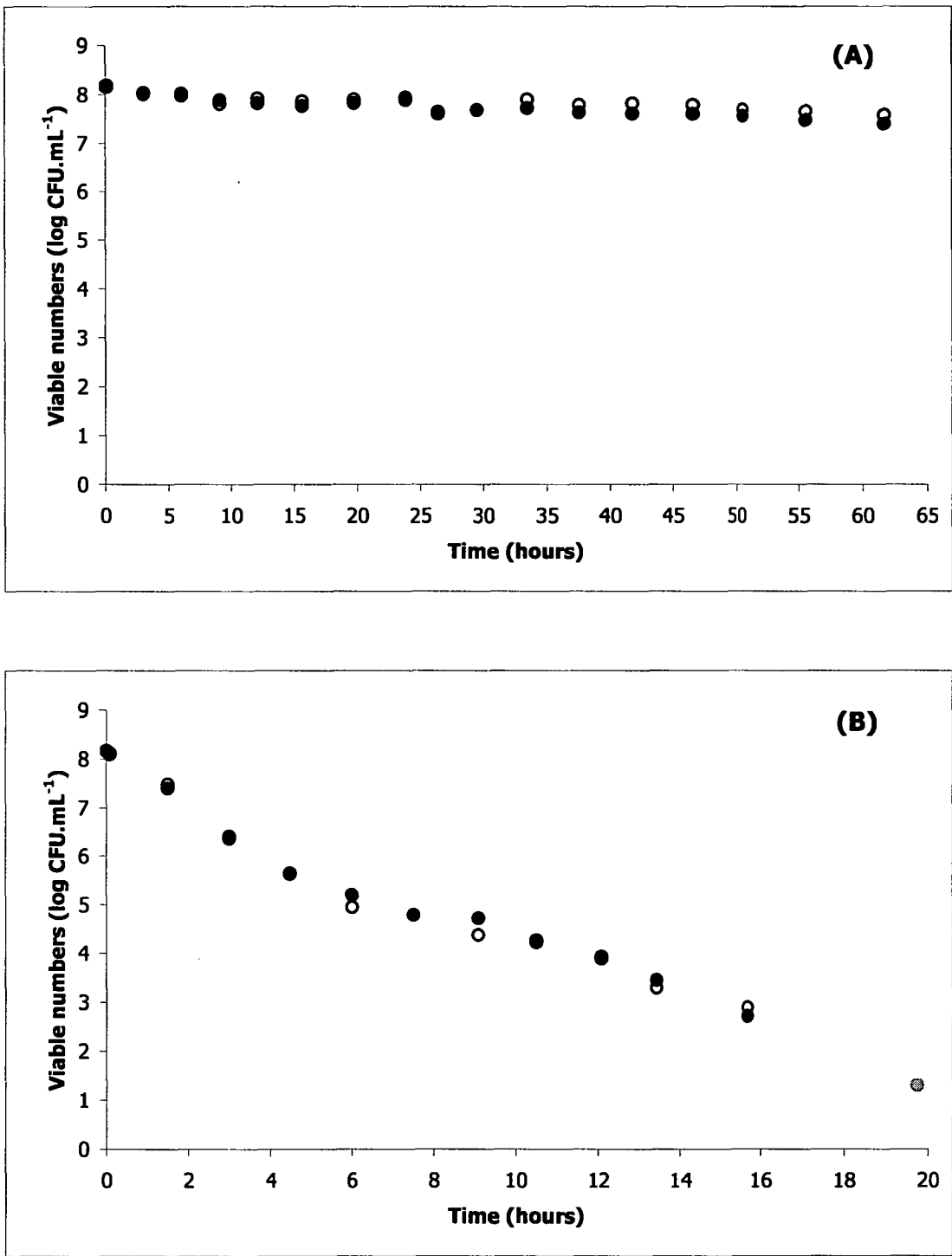
The 2-DE gels were analysed using PDQuest software to determine changes in protein expression due to the low  $a_w$  treatment. Specifically, tiff versions of the digitised gel images (6.2.3.5) were transformed, cropped and filtered. Spots were detected and matched automatically by the software and with manual intervention by the candidate. Spots were deemed to be valid (rather than background noise) if they were observed in two of the three replicate gels obtained for each treatment.

A 'master gel' image was generated to show and number all of the spots detected in any of the treatments and indicate their location and average intensity. The images were normalised to allow for differences in the volume of protein originally loaded to the gel. A protein spot present in the low  $a_w$ -treated samples but not in one or both of the controls (or vice versa) was taken to be a qualitative change in protein expression. Quantitative variance in protein expression between the  $a_w$ -treated sample and each of the controls was determined based on the spot intensity and Gaussian modelling using the PDQuest software. The same software was used to analyse significance by Student's  $t$  tests ( $P \leq 0.05$ ).

## **6.3 RESULTS**

### **6.3.1 Assessment of the Effect of Betaine on the Survival of *Escherichia coli* in Response to Lethal Water Activity**

The provision of exogenous betaine aids *E. coli* SB1 in their ability to multiply in growth limiting  $a_w$  environments (Krist, 1997). To determine if betaine also contributes to the survival of *E. coli* exposed to lethal  $a_w$ , the inactivation kinetics of *E. coli* M23 were determined in response to  $a_w$  0.90 solely and in combination with pH 3.5, in the presence and absence of 2 mM betaine. The results are given in Figure 6.2 and include survival curves that have been previously depicted in Figure 3.4 (Section 3.3.2.2). In response to both inimical treatments, the viability of *E. coli* M23 was not altered by the addition of 2 mM betaine to the medium. These findings were replicated in a separate experiment. Therefore, under the conditions examined, betaine availability does not affect the survival characteristics of osmotically stressed *E. coli* M23 populations.



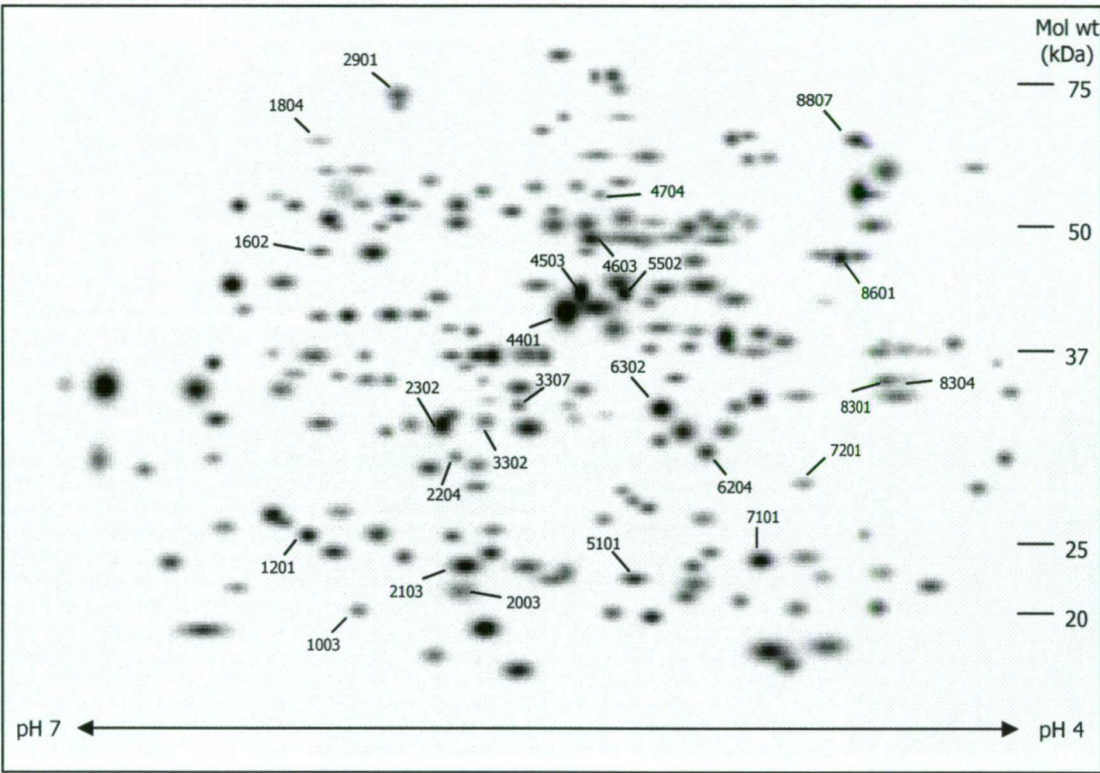
**Figure 6.2** The inactivation kinetics of *E. coli* M23 in response to (A)  $a_w$  0.90, or (B)  $a_w$  0.90 and pH 3.5 at 25°C in MM with (●) and without (○) 2 mM betaine. NaCl was used as the humectant and HCl as the acidulant. Grey data points indicate that the number of viable cells was below the minimum level of detection. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

### 6.3.2 Evaluation of Protein Expression in *Escherichia coli* that Survive Lethal Water Activity Stress

Subsequent experiments used proteomic methods to analyse protein expression in *E. coli* M23 that survive lethal  $a_w$  conditions for an extended time. Analyses focussed, in particular, on the expression of stress-related proteins. The results described below are based on a single experimental design, which was replicated three times. Proteins extracted from the untreated and low  $a_w$ -treated populations were separated in the first dimension according to the pI of the protein (over a pH range of 4 – 7) and then in the second dimension based on protein size. Computer-assisted analysis of the nine gels (which include three replicates of each treatment and are shown in Appendix D) generated a single synthetic image (master gel; given in Figure 6.3) that showed and numbered protein spots detected in all treatments. Approximately 209 distinct protein spots were detected.

A total of 93 spots were excised for tryptic in-gel digestion and MALDI-TOF MS analysis to determine the identity of those proteins. The peptide mass fingerprints generated were assigned to the complete proteomic database of *E. coli* (all strains) using NCBI and results were confirmed by subsequent searching of the SwissProt database. In total, 24 proteins were successfully identified by MALDI-TOF MS analysis (Table 6.2). For two pairs of the identified proteins, the spots were different isoforms of the same proteins that can be deamidated, phosphorylated, or modified by chemical groups that shift the pI toward an acidic pH (Liebler, 2002). Therefore, effectively 22 proteins were identified. The function of each of the identified proteins was determined by searching the UniProt protein knowledge database. The vast majority of the proteins identified are involved in various metabolic pathways in *E. coli*, whereas some are stress-related and others perform some other role within the cell.





**Figure 6.3** Master gel showing proteins extracted from populations of *E. coli* M23 left untreated or exposed to  $a_w$  0.90 and separated by 2-DE in the first dimension (horizontally) according to their pI (over pH 4 – 7) and in the second dimension (vertically) based on their size (over approximately 10 – 100 kDa). Molecular weight markers were run simultaneously and approximate indications are given on the right. The numbered protein spots were identified by peptide mass fingerprinting using MALDI-TOF MS. Numbers represent the spot numbers listed in Table 6.2.

**Table 6.2** Summary of proteins expressed in *E. coli*, identified by MALDI-TOF MS analyses and categorised according to their function (metabolic-related, stress-related or other).

| Spot<br>number    | Matched protein name  | Calculated |      | Mowse<br>score |
|-------------------|---|------------|------|----------------|
|                   |   | Size (Da)  | pI   |                |
| Metabolic-related |   |            |      |                |
| 1003              | Glutathione S-transferase   | 22 968     | 5.85 | 63             |
| 1201              | 2,3-Bispyrophosphoglycerate-dependent<br>phosphoglycerate mutase                                      | 28 408     | 5.86 | 141            |
| 1602              | Tryptophanase   | 53 098     | 5.88 | 139            |
| 1804              | Glutaminyl-tRNA synthetase  | 63 877     | 5.89 | 55             |
| 2003              | Glutamine-binding protein   | 24 933     | 5.99 | 70             |
| 2103              | Triosephosphate isomerase   | 27 126     | 5.64 | 54             |
| 2302              | Malate dehydrogenase  | 32 417     | 5.61 | 91             |
| 2901              | Formate acetyltransferase 1   | 85 581     | 5.69 | 66             |
| 3302              | Phosphoenol pyruvate-dependent sugar<br>phosphotransferase system mannose-specific<br>EIIAB component | 34 894     | 5.74 | 56             |
| 3307              | Phosphofructokinase   | 32 911     | 5.18 | 62             |
| 4503              | Enolase   | 45 552     | 5.32 | 173            |
| 4704              | Glutamate decarboxylase   | 37 154     | 5.14 | 65             |
| 5502              | (As for 4503)   |            |      | 195            |
| 7101              | Phosphoribosylaminoimidazole-<br>succinocarboxamide synthase  | 27 149     | 5.07 | 54             |
| 8301              | ADP-L-glycero-D-mannoheptose 6- epimerase   | 34 936     | 4.80 | 127            |
| 8304              | (As for 8301)   |            |      | 114            |
| 8601              | ATP synthase  | 50 220     | 4.90 | 206            |
| Stress-related    |   |            |      |                |
| 4401              | Elongation factor Tu  | 42 321     | 5.22 | 100            |
| 4704              | Glutamate decarboxylase   | 37 154     | 5.14 | 65             |
| 5101              | Stringent starvation protein A  | 24 215     | 5.22 | 76             |
| 8807              | Chaperone protein dnaK (heat shock protein)   | 68 998     | 4.83 | 82             |
| Other             |   |            |      |                |
| 2204              | Hypothetical protein yggE   | 26 611     | 6.21 | 124            |
| 4603              | Transposon Tn7 transposition protein  | 31 465     | 5.72 | 52             |
| 6204              | D-Galactose D-glucose binding protein   | 33 347     | 5.25 | 118            |
| 6302              | Outer membrane protein A precursor  | 37 292     | 5.99 | 58             |

Four of the identified proteins have been previously shown to be involved in the response of *E. coli* to specific stresses. Elongation factor Tu regulates the translation of proteins when the cell is deprived of nutrients (Weijland *et al.*, 1992), glutamate decarboxylase helps maintain the cytosolic pH in acidic environments (Castanie-Cornet *et al.*, 1999), and stringent starvation protein A confers transcriptional regulation and is usually synthesised in response to amino acid starvation and acid stress (ExPASy, 2005; Hansen *et al.*, 2005). Furthermore, expression of chaperone protein dnaK was observed. This protein regulates the folding of proteins in the cell in response to high temperature (Neidhardt and VanBogelen, 1987) and osmotic stresses (Meury and Kohiyama, 1991) and thereby preserves the capacity of those proteins to function under such stresses.

To determine if the expression of these stress-related proteins altered in response to the low  $a_w$  treatment, the nine 2-DE gels generated and described above were used for comparisons between osmotically stressed cultures and their unstressed counterparts. It should be noted that the present study used two control populations: the first was a proportion of the population used to inoculate the low  $a_w$ -broths (i.e. the inoculum control) and the second was derived from the same inoculum but was transferred to broth without added NaCl and so provided an aged but not osmotically stressed control. To constitute altered expression due to the osmotic stress, the level of protein expression in low  $a_w$ -treated cells was required to significantly ( $P \leq 0.05$ ) differ by at least two-fold to that in *both* of the control treatments. A comparison of the level of expression between the osmotically challenged population and both of the controls indicated that the expression of elongation factor Tu, glutamate decarboxylase, stringent starvation protein A and chaperone protein dnaK were not altered by the osmotic stress.

Given that these proteins did not alter in their expression due the osmotic stress, analyses continued to determine which proteins, if any, did change in their level of expression. Proteins that were present in the low  $a_w$ -treated *E. coli* M23 populations but not in either of the controls (qualitative variance) or at a significantly greater level than both of the controls (quantitative variance) were of primary interest. Such proteins are indicative of a unique response to the osmotic environment in *E. coli*. Equally important are proteins that decrease in their expression compared to both of the control treatments because this might identify processes that interfere with the cell's ability to cope with the osmotic stress. These processes might exacerbate the osmotic stress or might not be vital for survival and are, therefore, an inappropriate use of the cell's resources. Differences in protein expression between low  $a_w$ -treated *E. coli* and only one of the control populations provide an internal control on the method used but are not due to the  $a_w$  treatment *per se* and relate instead to differences in cell density or population age.

Of the 209 protein spots with molecular masses from around 10 – 100 kDa and a pI range from 4 to 7 (illustrated in Figure 6.2), 137 proteins (66%) were expressed at similar levels in the low  $a_w$ -treated and both of the untreated populations. The expression of 72 proteins (34%) showed significant alterations ( $P \leq 0.05$  and at least two-fold difference) between the low  $a_w$ -treated populations and one or both of the control treatments, as shown in Table 6.3. Eleven proteins (5%) altered in their expression due to the osmotic stress (i.e. differed between the low  $a_w$ -treated population and both of the control populations) as indicated in the shaded cells of Table 6.3.

**Table 6.3**      The number of proteins in *E. coli* M23 that increased or decreased in their expression in response to treatment with  $a_w$  0.90 for approximately 200 hours, compared to the inoculum (ino) or aged control, or both (ino + aged), as determined by 2-DE methodologies.

|                       | Increased  |             |                   | Decreased  |             |                   |
|-----------------------|------------|-------------|-------------------|------------|-------------|-------------------|
|                       | <i>ino</i> | <i>aged</i> | <i>ino + aged</i> | <i>ino</i> | <i>aged</i> | <i>ino + aged</i> |
| <b>Total</b>          | 9          | 26          | 4                 | 11         | 38          | 7                 |
| - <i>Qualitative</i>  | 1          | 3           | 0                 | 10         | 31          | 7                 |
| - <i>Quantitative</i> | 8          | 23          | 4                 | 1          | 7           | 0                 |

The expression of only four proteins was observed to increase in cells that survived the osmotic stress for approximately 200 hours. These proteins were all expressed in the control populations but their expression increased significantly ( $P \leq 0.05$ ) and greater than two-fold in the osmotically stressed populations. The identity of one of those proteins, glutaminyl-tRNA synthetase, which is involved in amino acid metabolism, was determined by MALDI-TOF analysis. Conversely, the levels of seven proteins were determined to decrease due to the osmotic stress. In that instance, all of the proteins varied qualitatively (i.e. they were not expressed in the low  $a_w$ -treated cells but were evident in both of the control populations). Three were identified: glutathione S-transferase, formate acetyltransferase 1, and D-galactose D-glucose binding protein.

**6.4 DISCUSSION**

The ability of bacteria to survive or even grow in environments characterised by adverse conditions is widely held to rely upon the ability of microorganisms to respond to such inimical constraints. Stress responses typically involve complex mechanisms that ultimately reverse or minimise the detrimental effect of the stress and ensure that cellular functioning can continue, albeit at a reduced level

(reviewed by Storz and Hengge-Aronis, 2000). The specific response of *E. coli* to growth limiting, non-lethal osmotic stress has been well described (Csonka, 1989; Galinski and Truper, 1994; Gutierrez *et al.*, 1995; Csonka and Epstein, 1996) and clearly demonstrates that bacteria respond to this mild constraint in a manner that allows cell growth to continue (Krist, 1997; Krist *et al.*, 1998). However, very few investigations have studied the physiology of bacteria exposed to the analogous *lethal* constraint (Shadbolt *et al.*, 2001; Brown, 2002) and, therefore, direct evidence for a specific response that aids in cell *survival* remains to be shown.

While essentially providing a mechanistic interpretation of bacterial inactivation, the identification of a specific response initiated by bacteria to enhance their survival in inimical osmotic environments has some practical implications. The ability to inhibit or prevent that response in bacteria that contaminate foods would result in their enhanced inactivation and reduce the likelihood of foodborne illness. Importantly, the identification of such a response, if one does occur, is made difficult by the lack of knowledge as to the actual mechanisms that mediate low  $a_w$ -induced inactivation of *E. coli*. As a result, the processes by which *E. coli* might resist that lethal effect, and thereby improve their chance of survival, can only be speculated on and there are many options that require investigation. The current work was directed at assessing the involvement of *previously described* stress responses in the survival of *E. coli* M23 exposed to lethal  $a_w$  stress.

#### **6.4.1 Assessment of the Effect of Betaine on the Survival of *Escherichia coli* in Response to Lethal Water Activity**

In *E. coli*, compatible solute accumulation forms an essential part of the osmoregulatory response to growth limiting  $a_w$ . By maintaining the necessary

turgor for cell division to occur, the accumulation of compatible solutes reduces the minimum  $a_w$  in which bacteria can grow (Krist, 1997). Further to the maintenance of cell turgor, compatible solutes protect macromolecules under conditions of osmotic stress. Their accumulation, as an alternative to ionic salts, reduces protein denaturation (Warr *et al.*, 1984; Arakawa and Timasheff, 1985), and also stabilises macromolecules by altering the structure of water immediately surrounding those molecules (Arakawa and Timasheff, 1983). The candidate hypothesised that, based on the protective effect of compatible solutes, their accumulation might also improve the capacity of *E. coli* to survive in environments characterised by lethal  $a_w$  and, thus, initial experiments were undertaken to test this hypothesis.

An evaluation of the inactivation kinetics of *E. coli* M23 exposed to  $a_w$  0.90 (or in combination with pH 3.5) in the presence and absence of betaine indicated that betaine did not alter the survival characteristics of *E. coli* M23. It could not be determined from this investigation whether the stressed cells accumulated the exogenously available betaine, only that, if they did, this process did not improve cell survival. Thus, if accumulation of compatible solutes occurs it does not constitute a stress response that accounts for the ability of some *E. coli* M23 cells to survive in lethal  $a_w$  environments. Importantly, the accumulation of betaine is an active process and, in a study that measured the uptake of radiolabelled acetic acid as a sole energy source in *E. coli* M23 populations exposed to lethal  $a_w$ , Shadbolt *et al.* (2001) demonstrated that these cells take up and metabolise little energy-providing substrates following exposure to that inimical condition. Therefore, while experiments could have been undertaken to measure the uptake of exogenously supplied and radiolabelled betaine by *E. coli* exposed to lethal  $a_w$ , the results of Shadbolt *et al.* (2001) and the current study collectively suggest that active uptake is unlikely.

To describe more conclusively the involvement, or lack thereof, of other (i.e. non-compatible solute related) osmoregulatory-associated processes in the response of *E. coli* to lethal  $a_w$  environments further experimentation is required. Prospective experiments might analyse the expression of specific genes or proteins previously determined to change during osmoregulation at growth-permitting  $a_w$  values when *E. coli* is exposed to lethal  $a_w$  conditions. Potential candidate proteins include the outer membrane porins OmpF and OmpC and constituents of the potassium uptake system Kdp. Assessment of their expression could be by western blot analyses (where the appropriate antibodies are available) or reverse-transcription PCR. Another, more indirect, means by which the expression of stress-related proteins can be examined is by using proteomic methods, which allow the expression of multiple proteins to be assessed using a single protocol. Such an approach is often a useful starting point in the study of cellular physiology because the results may indicate more specific areas for future research. Therefore, subsequent investigations evaluated protein expression in *E. coli* M23 that survive lethal  $a_w$  environments for an extended period, in an attempt to identify stress-related proteins.

#### **6.4.2 Evaluation of Protein Expression in *Escherichia coli* that Survive Lethal Water Activity**

Proteomic studies provide a description of the cellular proteome and, therefore, afford a means to assess the expression and identity of multiple proteins at once. Although proteomic approaches have been used to determine the immediate response of *E. coli* to growth-limiting but non-lethal osmotic stress (Clark and Parker, 1984; Botsford, 1990; Hengge-Aronis *et al.*, 1993), this work is the first to study protein expression in *E. coli* exposed to lethal  $a_w$ , where only a small



proportion of the original population has survived. By separating proteins using 2-DE, a method that has become synonymous with proteomic applications (Lopez, 2000), identifying those proteins, as far as possible, using MALDI-TOF MS techniques and comparing protein expression in control and low  $a_w$ -treated populations of *E. coli* M23, the involvement of stress-related proteins was assessed.

The expression of four proteins, previously reported to be involved in stress responses in *E. coli*, was identified in cells that survived exposure to lethal  $a_w$ . Those were elongation factor Tu, glutamate decarboxylase, stringent starvation protein A and chaperone protein dnaK. Only dnaK has been implicated in the response of *E. coli* to growth-limiting osmotic shock (Meury and Kohiyama, 1991), and it also functions in response to high temperature (Neidhardt and VanBogelen, 1987). The other stress-related proteins identified function as part of the cellular response to starvation and/or acid stress (Weijland *et al.*, 1992; Castanie-Cornet *et al.*, 1999; Hansen *et al.*, 2005). Comparison of the level of expression of each of these proteins in all treatments indicated that these proteins were expressed in non-osmotically stressed, stationary phase populations of *E. coli* M23 and that expression did not alter due to application of the low  $a_w$  stress. Therefore, the involvement of these proteins as a part of a specific response in *E. coli* M23 to lethal  $a_w$  conditions was ruled out.

From the same experiment, the expression of 11 proteins was observed to alter in response to treatment with lethal  $a_w$  conditions. Four of those were able to be identified by MALDI-TOF MS analyses and were found to be glutathione S-transferase (a central intermediary in cellular metabolism), formate acetyl transferase 1 (that catalyses the non-oxidative conversion of glucose), D-galactose D-glucose binding protein (a transport protein) and glutaminyl-tRNA synthetase

(involved in amino acid metabolism) (ExPASy, 2005). The former three were expressed in control populations of *E. coli* M23 but not in those cells that were exposed to low  $a_w$  conditions. Their altered expression represents an adjustment in the functioning of the cell. In contrast, the expression of glutaminyl-tRNA synthetase increased in response to the lethal  $a_w$  stress. It is a cytosolic enzyme that activates L-glutamine and transfers it to specific transfer RNA molecules in the first stage of protein biosynthesis (ExPASy, 2005). At present, there is no evidence to suggest that this protein functions in *E. coli* to enhance survival in inimical environments and it is difficult to speculate how it might. It is more likely that the increased expression of this protein occurs as a consequence of the low  $a_w$  treatment rather than as a means to counteract its detrimental impact.

Seven proteins that altered in their expression due to the osmotic challenge were not successfully identified by MALDI-TOF MS analyses, despite these tests being performed on at least two separate occasions. This perhaps reflects inadequate separation of the protein mixture. If so, spots subjected to trypsin digestion would contain multiple proteins, thus reducing the likelihood of successfully matching the peptide mass fingerprint by database searching. Because these proteins might function as a stress response in *E. coli*, their identification is important. Future work should attempt to determine their identities by MALDI-TOF MS techniques by improving separation of the sample, i.e. using IPG strips of a narrower pH range either alone, or in conjunction with prefractionation using a liquid-phase electrophoretic system (Herbert and Righetti, 2000).

The current proteomic-based investigation has excluded four stress-related proteins as specific responses to lethal  $a_w$  and, moreover, has found no direct evidence for a stress-related response in *E. coli* M23 that aids the cells in surviving low  $a_w$  stress.

Using this method, however, it is impossible to detect all of the proteins expressed by *E. coli* at the time of sampling and, thus, other stress-related proteins may have been overlooked. For example, although 2-DE is the best separation technique currently available for protein expression profiling, it is unable to resolve and display all of the proteins within a proteome because stains used for visualisation detect only the most abundant proteins within the sample (Gygi *et al.*, 1999; Gygi *et al.*, 2000). Further, some proteins (typically the larger and more hydrophobic ones) show marginal solubility and are not well visualised on 2-DE gels (Wilkins *et al.*, 1998). The evaluation of the proteome was further limited, in the current work, to those proteins with a pI between pH 4 and 7, due to separation in the first dimension over this pH range. Therefore, any proteins that:

- have a pI less than pH 4 or greater than pH 7,
- are expressed at low levels,
- are rapidly turned-over, or
- have other characteristics that make their separation and staining by proteomic methods difficult,

including any osmotically-induced proteins that function to aid *E. coli* in their survival in response to low  $a_w$  conditions, could not be detected in the current investigation, or indeed in most proteomic experiments.

The limitations inherent in proteomic methodologies could be partly overcome by combining the results of the current investigation with large-scale genomic studies (e.g. using DNA microarray technology). The latter approach uses gene-specific oligonucleotides immobilised on solid substrates to probe complex nucleic acid samples and determine the expression of thousands of genes at once (Heller, 2002). This method allows the change in expression of all of the representative genes to be definitively stated. Coupling of protein expression analyses with DNA

microarray technology allows the transcription of those genes into proteins (which carry out the function of the gene) to be confirmed, and is a tactic that is being increasingly used to study cellular physiology on a large-scale (Pandey and Mann, 2000; Yoon *et al.*, 2003; Hansen *et al.*, 2005). Therefore, future experiments that use DNA microarray techniques to assess the physiology of low  $a_w$ -treated *E. coli* could more comprehensively confirm the findings of this proteomic-based study, that *E. coli* does not initiate a specific stress response to survive in lethal  $a_w$  environments.

### **6.4.3 Conclusions**

From the investigations presented in this chapter, it appears that compatible solute accumulation is unlikely to constitute a stress response to ensure survival in cells exposed to potentially lethal  $a_w$  stress. Further, it is apparent that the stress-related proteins elongation factor Tu, glutamate decarboxylase, stringent starvation protein A and chaperone protein dnaK do not form a specific response to lethal osmotic stress in *E. coli* M23. Other proteins do alter in their expression but they are more likely to constitute a change caused by the inimical environment rather than as a means of reversing or minimising the detrimental effect that leads to inactivation. Therefore, this work found no direct evidence for a specific stress response initiated by *E. coli* M23 to enhance their survival in lethal  $a_w$  environments suggesting that the inactivation processes are not actively resisted by the cells. The results presented will form a basis for interpretation of studies that more comprehensively investigate the physiological response of *E. coli* to lethal  $a_w$  values using techniques such as DNA microarray to further clarify the potential involvement of specific stress responses in *E. coli* exposed to lethal  $a_w$  stress.

## **Chapter 7**

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### **Thesis Summary**

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The primary objective of the present study was to enhance understanding of the processes of low water activity-induced inactivation of *E. coli*. Initially, the kinetics of inactivation of *E. coli* in response to low water activity environments were described. Later investigations provided insight into the mechanisms responsible for the lethal effect of low water activity and of the physiology and physiological responses of cells that are able to survive in low water activity environments.

Initially, factors that influence the quantification of bacterial viability and injury were evaluated (Chapter 2) and, from this, materials were selected and methods developed to reduce experimental artefacts. Specifically, a protocol for the preparation of experimental inocula and a specific suspension medium were developed to provide an accurate and reproducible assessment of the loss of viability in *E. coli* M23 populations exposed to low water activity, low pH or both. Further, standard enumeration methods were found to be inadequate for the quantification of injury in *E. coli* M23 populations subjected to low water activity environments. Thus, methods were developed to optimise the recovery of the uninjured proportion of the population.

Using the methodologies developed, the kinetics of low water activity-induced inactivation of *E. coli* were described (Chapter 3). *E. coli* M23 populations exposed to low water activity constraints exhibited up to three distinct phases of inactivation, including an initial phase of rapid inactivation that was followed by a second phase with a slower inactivation rate and then a third phase. That final phase was characterised by a more rapid rate of inactivation that persisted until the number of viable cells was below the minimum level of detection. Inactivation studies often report phases of inactivation analogous to the first and second phases described in

the present work. However, the third and more rapid phase of inactivation has not been widely observed. That phase is easily overlooked in studies that do not continue to measure viability at low population densities. In a practical sense, the ability to induce the third and more rapid phase of inactivation during food manufacturing processes would better ensure the elimination of foodborne bacteria and contribute to a safer product.

Because the preservation of foods commonly relies on a combination of inhibitory factors, the effect of simultaneously exposing *E. coli* to low water activity and low pH stresses was evaluated. The response was highly dependent on the suspending medium employed. Further, when the same stresses were applied independently (i.e. as an initial and subsequent stress), the order of application of the stresses influenced the decline of the *E. coli* population. Specifically, *E. coli* M23 that survived exposure to low water activity for 24 hours were better able to survive an additional acid challenge than when the order of stresses was reversed. This observation may have application in the design of more effective food processing regimes. Further investigations described the level of injury in *E. coli* M23 caused by low water activity or low pH, which illustrated that the effect of these stresses on *E. coli* M23 involve distinct processes.

The kinetics of inactivation of *E. coli* in response to conditions representative of Australian-produced uncooked, comminuted fermented meat products were presented (Chapter 4). Using a broth-based system, temperature was identified to be the most important variable controlling the loss of viability of *E. coli* M23. Water activity also had a strong effect, however, incorporation of that term into a predictive model actually reduced its predictive capacity when compared to uncooked, comminuted fermented meat-derived data. Thus, it was apparent that

the broth-based system did not accurately mimic the inactivation of *E. coli* in uncooked, comminuted fermented meat, but it did allow general patterns of inactivation to be addressed. The quantitative knowledge of *E. coli* inactivation developed in this work was used to generate a predictive model that has been employed within the Australian food industry and is an example of the applicability of such studies to the production of microbiologically safe foods.

Having described the kinetics of inactivation of *E. coli* by low water activity, investigations turned to an evaluation of (a) the mechanism(s) of that lethal action and (b) the physiology of cells able to survive in low water activity environments for an extended period. It was noted that while much information relating to the response of *E. coli* to growth-limiting water activity (i.e. a 'mild' stress) has been presented in the literature, relatively few descriptions are available to ascertain the effect of the analogous *lethal* constraint on *E. coli*.

An important development in the understanding of bacterial death was the identification of specific genetic modules (including *mazEF*) that mediate the death of bacteria in response to various stresses. As detailed in Chapter 5, the *mazEF* module appeared to mediate the inactivation of a proportion of *E. coli* MG1655 populations exposed to water activity 0.90, although this finding was considered ambiguous due to variation in the survival characteristics of two  $\Delta mazF$  *E. coli* mutants that, theoretically, should have behaved similarly. As a result, the mechanism of cell death in *E. coli* MG1655 exposed to  $a_w$  0.90 could not be conclusively shown to involve the *mazEF* system but nor could its association be ruled out. The potential involvement of this 'suicide' pathway did not alter the subsequent survival characteristics of the population, indicating that the programmatic removal of some cells within the population does not aid the



remaining cells in their ability to maintain essential cell functioning. Further, *mazEF* accounted for the inactivation of only a proportion of the *E. coli* MG1655 population and, thus, is not the sole mediator of cell death in response to low water activity conditions. Future experiments might use repair-deficient *E. coli* mutants to determine if damage to specific cellular components (e.g. DNA, RNA or protein molecules) also contributes to the toxicity of low water activity in *E. coli*.

In the final chapter, a series of investigations that attempted to identify a specific response to lethal water activity stress in *E. coli* were described. Although accumulation of the compatible solute betaine aids *E. coli* in their ability to grow following exposure to growth-limiting but non-lethal water activity, results of the current work indicate that the provision of this osmoprotectant does not enhance the survival of *E. coli* M23 treated with the analogous *lethal* constraint. Therefore, the accumulation of compatible solutes appears unlikely to represent a stress response that ensures *E. coli* survival in potentially lethal water activity environments. From analyses of protein expression in populations of *E. coli* M23 exposed to lethal water activity it was concluded that the stress-related proteins elongation factor Tu, glutamate decarboxylase, stringent starvation protein A and chaperone protein dnaK do not constitute an osmotically-induced stress response in *E. coli* M23. Overall, this work found no direct evidence for a specific stress response initiated by *E. coli* M23 to enhance their survival in lethal water activity environments. This suggested that the survival of *E. coli* in low water activity environments does not rely upon an active and stress-specific response (typical during exposure to mild water activity stress) but might be a passive process, determined purely by 'chance'. A series of experiments to address this issue were suggested for future investigations.

In conclusion, the present research has contributed to knowledge and understanding of the quantitative features of low water activity-induced inactivation of *E. coli*. Furthermore, this study has provided some insights into the mechanism of non-thermal inactivation and the response of *E. coli* to lethal stress, and identified avenues for further studies to reveal the mechanisms of non-thermal inactivation of *E. coli* by inimical osmotic challenges.

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# Appendices

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## APPENDIX A: Materials and Equipment

### A.1 MATERIALS

#### A.1.1 Bacterial Strains

*E. coli* strains used throughout the course of this study are described in Table A.1. Colonies taken from original plate culture were resuspended in nutrient broth containing 30% glycerol and stored in duplicate at  $-80^{\circ}\text{C}$ . One culture was used for the preparation of experimental inocula while the other was held in reserve. To prepare experimental inocula, cells were removed from the surface of the thawed stock culture using a sterile yellow pipette tip, plated to TSA (containing an antibiotic if required) and incubated for 14 hours at  $30^{\circ}\text{C}$  (for strains carrying plasmids pKD46 or pCP20) or  $37^{\circ}\text{C}$  (all other strains).

**Table A.1** *E. coli* strains used during the current study.

| Strain/Plasmid   | Description  | Reference/Source                  |
|------------------|--|-----------------------------------|
| BT340            | DH5 $\alpha$ , contains plasmid pCP20              | Cherepanov and Wackernagel (1995) |
| BW25141          | K12, for maintenance of pKD4                       | Datsenko and Wanner (2000)        |
| M23 <sup>a</sup> | OR:H-, non-pathogenic                              | Brown <i>et al.</i> (1997)        |
| MG1655           | K12, non-pathogenic, with or without plasmid pKD46 | Datsenko and Wanner (2000)        |
| N1               | MG1655, <i>mazF::kan</i>                           | This work                         |
| SC30             | MG1655, $\Delta mazF$                              | Christensen <i>et al.</i> (2003)  |
| SM01             | MG1655, $\Delta mazF$                              | This work                         |

<sup>a</sup> See Section 2.2.1 for a more detailed description of this strain.

#### A.1.2 Reagents

##### *Culture-Based Studies*

Bacteriological peptone, bile salts No. 3, tryptone, yeast extract and all broths were obtained from Oxoid (Australia), except for minimal davis broth without dextrose which was supplied by Difco Laboratories (USA). Agar (Grade J3) was supplied by Gelita (Australia). BDH (Australia) supplied the

standard pH solutions and Vaseline (Australia) the petroleum jelly. Many reagents were obtained from Sigma (USA) including betaine (hydrochloride; > 99% purity), D-glucose (> 99% purity), glycerol (~ 99% purity), paraffin oil, pyruvate (sodium salt; > 99% purity), resazurin (sodium salt; ~ 80% dye content) and sodium thioglycolate (> 99.5% purity). Sodium chloride (99% purity) was from Chem-Supply (Australia) and hydrochloric acid (31.5% purity), lactic acid (85 – 90% purity) and sodium hydroxide pellets from Ajax Chemicals (Australia). Antifreeze was acquired from BP (Australia).

### ***Molecular Biology Studies***

Oligonucleotide primers were synthesised by GeneWorks (Australia). Primers ChpAK1 and ChpA2 were purified by high-performance liquid chromatography. The HotStart Master Mix was from Qiagen (USA), which also supplied the QIAprep Spin Miniprep and QIAquick Purification kits. The DNA molecular weight markers (Hyperladder 1) were obtained from Bioline (USA) and the dideoxy terminator cycle sequencing mix from Beckman Coulter (USA). Tris acetic acid buffer was from Amresco (USA) and acetic acid from Ajax Chemicals (Australia). Sigma (USA) supplied the majority of reagents including agarose, ampicillin (sodium salt), L-arabinose (> 99% purity), bromophenol blue (sodium salt, 95% dye content), ethidium bromide (~ 95% purity), EDTA, guanidine hydrochloride (99% purity), kanamycin (sulphate salt), magnesium chloride (> 98% purity), magnesium sulphate (> 99% purity), potassium chloride (~ 99% purity), sucrose (> 99% purity), and xylene cyanol (~ 75% dye content).

### ***Proteomic Studies***

The Destreak™ reagent was from Amersham Biosciences (USA) and trypsin (porcine sequencing grade) was from Promega (USA). Sigma (USA) supplied acetonitrile (> 99.5% purity), amidosulfobetaine-14, ammonium bicarbonate (> 99% purity), benzonase (> 90% purity), bovine plasma  $\gamma$ -globulin, iodoacetamide (> 99% purity), potassium phosphate (> 99% purity), sodium dodecyl sulphate (> 98.5% purity), sodium phosphate (> 98% purity), trifluoroacetic acid (> 99% purity) and urea (> 98% purity). Dithiothreitol was from Astral Scientific (USA). The ethanol and methanol were from Ajax Chemicals (Australia). The remaining reagents (agarose with low melting point, mineral oil, the *RCDC*™ protein assay kit, sequential extraction reagents 1 and 3, SYPRO ruby protein gel stain, tributylphosphine, tris glycine sodium dodecyl sulphate buffer, tris hydrochlorate buffer) were obtained from BioRad (USA) as were the Criterion™ 12.5% tris hydrochlorate resolving gels, the ReadyStrip™ IPG strips and the protein unstained standards (Precision Plus proteins™).

### A.1.3 Bacteriological Media

The bacteriological media used throughout the course of this study were prepared and stored according to the manufacturer's instructions (Oxoid, [www.oxoid.com](http://www.oxoid.com)), or as described below.

#### ***Brain Heart Infusion Agar with 0.1% sodium pyruvate (BHA-P)***

|                            |      |
|----------------------------|------|
| Brain heart infusion broth | 37 g |
| Agar (1.5%)                | 15 g |
| Sodium pyruvate (0.1%)     | 1 g  |
| dH <sub>2</sub> O          | 1 L  |

Autoclaved (121 °C, 20 minutes).

#### ***Brain Heart Infusion Agar with 0.15% bile salts (BHA-BS)***

|                            |       |
|----------------------------|-------|
| Brain heart infusion broth | 37 g  |
| Agar (1.5%)                | 15 g  |
| Bile salts (0.15%)         | 1.5 g |
| dH <sub>2</sub> O          | 1 L   |

Autoclaved (121 °C, 20 minutes). BHA-BS was stored in the dark until use.

#### ***Brain Heart Infusion Broth (BHI)***

|                            |      |
|----------------------------|------|
| Brain heart infusion broth | 37 g |
| dH <sub>2</sub> O          | 1 L  |

#### ***Cooked Meat Medium (CMM)***

|                    |       |
|--------------------|-------|
| Cooked meat medium | 100 g |
| dH <sub>2</sub> O  | 1L    |

200 mL aliquots of solution were stomached for 1 minute and filtered using Grade 1 filter paper of 11 µm pore size to remove the insoluble pellets.

#### ***Luria-Bertani Broth (LB)***

|                   |      |
|-------------------|------|
| Tryptone          | 10 g |
| Yeast extract     | 5 g  |
| NaCl              | 10 g |
| dH <sub>2</sub> O | 1 L  |

Autoclaved (121 °C, 20 minutes).



**Minimal Minerals Medium with 0.1% D-Glucose (MM)**

|                                      |        |
|--------------------------------------|--------|
| Minimal Davis broth without dextrose | 10.6 g |
| dH <sub>2</sub> O                    | 1 L    |

Autoclaved (121°C, 20 minutes). The medium was allowed to cool prior to the addition of 10 mL 10% D-glucose solution (0.1%).

**Minimal Minerals Medium with 0.1% D-Glucose and 2 mM betaine (MM-B)**

MM was prepared as described above and 1 mL of a 2 M filter sterilised betaine solution (2 mM) was added.

**Nutrient Broth (NB) ± 30% glycerol**

|                   |      |
|-------------------|------|
| Nutrient broth    | 13 g |
| dH <sub>2</sub> O | 1 L  |

Autoclaved (121°C, 20 minutes). When required, 300 mL glycerol was added prior to the autoclaving to give nutrient broth with 30% glycerol.

**Recovery broth**

|                      |        |
|----------------------|--------|
| Tryptone (2%)        | 10 g   |
| Yeast extract (0.5%) | 2.5 g  |
| NaCl (10 mM)         | 0.29 g |
| KCl (2.5 mM)         | 0.09 g |

Reagents were combined and the volume made to 500 mL with dH<sub>2</sub>O. The solution was autoclaved (121°C, 20 minutes) and allowed to cool to RT. Magnesium ions were added to a final concentration of 20 mM by the addition of 5 mL of a 2 M filter sterilised stock solution (i.e. 1 M MgCl<sub>2</sub> and 1 M MgSO<sub>4</sub> in dH<sub>2</sub>O). To 99 mL of this solution, glucose was added to a final concentration of 20 mM by the addition of 1 mL 2 M filter sterilised glucose solutions (prepared in dH<sub>2</sub>O). The solution was stored at 4°C until use.

**Tryptone Soya Agar (TSA) ± antibiotics**

|                     |      |
|---------------------|------|
| Tryptone soya broth | 30 g |
| Agar (1.5%)         | 15 g |
| dH <sub>2</sub> O   | 1 L  |

Autoclaved (121°C, 20 minutes). Where appropriate, selective agents were filter sterilised and added to molten TSA cooled to 55°C. Ampicillin was used at a final concentration of 100 µg/mL and kanamycin at 25 µg/mL.

***Tryptone Soya Broth (TSB) ± antibiotics or inducing agents***

|                     |      |
|---------------------|------|
| Tryptone soya broth | 30 g |
| dH <sub>2</sub> O   | 1 L  |

Autoclaved (121°C, 20 minutes). Where appropriate, selective or inducing agents were filter sterilised and added to TSB at RT. Ampicillin was used at a final concentration of 100 µg/mL, kanamycin at 25 µg/mL and L-arabinose at 1 mM.

**A.1.4 Solutions**

Solutions were stored at RT unless otherwise stated.

***Agarose solution***

- For proteomic studies

|                                   |        |
|-----------------------------------|--------|
| Agarose, low melting point (0.5%) | 0.5 g  |
| 1X TGS buffer                     | 100 mL |
| 0.1% Bromophenol blue (0.001%)    | 1 mL   |

Agarose was dissolved in the buffer with heating and then bromophenol blue was added for colour.

- For molecular biology studies

|              |        |
|--------------|--------|
| Agarose (1%) | 1 g    |
| 50X TAE (1X) | 2.0 mL |

Reagents were combined and the volume adjusted to 100 mL with dH<sub>2</sub>O. A 200 µL volume of 1% ethidium bromide was added to 20 mL of the agarose solution immediately prior to use.

***Diluent***

|                                |       |
|--------------------------------|-------|
| Bacteriological peptone (0.1%) | 1 g   |
| NaCl (0.85%)                   | 8.5 g |
| dH <sub>2</sub> O              | 1 L   |

Autoclaved (121°C, 20 minutes).

***Equilibration base buffer***

|  |        |
|--|--------|
| Urea (6 M)   | 36 g   |
| 20% Sodium dodecyl sulphate (2%)                     | 10 mL  |
| 1.5 M Tris hydrochlorate buffer, pH 8.8,<br>(0.05 M) | 3.3 mL |
| 50% Glycerol (20%)                                   | 40 mL  |

Reagents were combined and the volume adjusted to 100 mL with Milli-Q H<sub>2</sub>O. Aliquots (0.5 mL) were stored at -20°C.

**Gel storage solution**

|                |        |
|----------------|--------|
| Ethanol (40%)  | 400 mL |
| Glycerol (10%) | 100 mL |

Reagents were combined and the volume made up to 1 L with Milli-Q H<sub>2</sub>O.

**Loading buffer**

|                  |          |
|------------------|----------|
| Bromophenol blue | 0.0125 g |
| Xylene cyanol    | 0.125 g  |
| Sucrose          | 20.0 g   |

Reagents were combined and the volume adjusted to 50 mL with dH<sub>2</sub>O. The solution was stored at 4°C.

**Low salt buffer**

|   |        |
|---|--------|
| KCl (3 mM)  | 0.22 g |
| KH <sub>2</sub> PO <sub>4</sub> (1.4 mM)            | 0.19 g |
| NaCl (68 mM)  | 4.0 g  |
| NaH <sub>2</sub> PO <sub>4</sub> , anhydrous (9 mM) | 1.1 g  |

Reagents were combined and Milli-Q H<sub>2</sub>O added to a final volume of 1 L. The solution was autoclaved (121°C, 20 minutes) and stored at 4°C.

**Multiple surfactant solution (MSS)**

|   |        |
|---|--------|
| Sequential extraction reagent 3         | 0.5 mL |
| 1 mg/mL Amidosulfobetaine-14 (10 µL/mL) | 5 µL   |
| 200 mM Tributylphosphine (2 mM)         | 5 µL   |

Reagents were combined immediately prior to use and the solution mixed by pipetting. When required, the tributylphosphine component was replaced with 6 µL of Destreak™ reagent.

**SYPRO Ruby fixative**

|                  |        |
|------------------|--------|
| Methanol (10%)   | 100 mL |
| Acetic acid (7%) | 70 mL  |

Reagents were combined and made up to a final volume of 1 L with Milli-Q H<sub>2</sub>O.

**Tris acetic acid EDTA buffer (50X)**

|                                 |         |
|---------------------------------|---------|
| Tris acetic acid buffer (40 mM) | 242 g   |
| Acetic acid                     | 57.1 mL |
| 0.5 M EDTA, pH 8.0 (1 mM)       | 100 mL  |

Reagents were combined and the volume made up to 1 L with dH<sub>2</sub>O. Before use, the solution was diluted to 1X in dH<sub>2</sub>O.

**Trypsin solution**

|                   |        |
|-------------------|--------|
| Trypsin (0.01%)   | 20 µg  |
| 50 mM Acetic acid | 200 µL |

Reagents were combined and aliquots (25 µL) were stored at -80°C. Immediately prior to use, a 25 µL aliquot was activated by the addition of 450 µL of 50 mM ammonium bicarbonate (pH 8.0).

**A.2 EQUIPMENT****Autoclave**

Pressure cooker RY-150 from Rinnai (Australia) used with countdown timing systems.

**Centrifuges**

1. Universal 16A, Imbros, Australia.
2. Microcentrifuge 5417R, Eppendorf, Germany.
3. Sorvall® SUPER T21, Kendro, USA.
4. Hettich Zentrifugen EBA12, HD Scientific, Australia.
5. J2-21M/E, Beckman Coulter, Australia.

**Electrophoresis cells**

1. Horizon®58 Life Technologies Horizontal Gel Electrophoresis Apparatus from Gibco BRL, USA.
2. Criterion™ cell (2 gel capacity) from BioRad Laboratories, USA.
3. Criterion™ Dodeca™ cell (12 gel capacity) from BioRad Laboratories, USA.

**Electroporator**

*E. coli* Pulser from BioRad, USA.

**Freezer (-80 °C)**

Ultra-low temperature freezer MDF-U50V from Sanyo, Japan.

**Image scanners**

1. CASBA™ 4 from Spiral Biotech, USA was employed to acquire images of bacterial colonies on agar plates.
2. Storm840 from Amersham Biosciences, USA that acquired fluorescent images of protein gels.

**Incubators**

1. Water bath (Model SWB20D) from Ratex Instruments, Australia used with a refrigeration unit (Model RC2) also from Ratex Instruments, Australia. An antifreeze solution (1:1 with dH<sub>2</sub>O) was used in this water bath, which was accurate to within  $\pm 0.1^{\circ}\text{C}$  of the set temperature. Incubations were static or with shaking as required.
2. Temperature-gradient incubator (model TN-2148) from Advantec MFS, USA. Incubations were conducted with shaking.

**Isoelectric focussing (IEF) cell**

PROTEAN<sup>®</sup> from BioRad Laboratories, USA used with a twelve strip capacity IEF tray obtained from the same company.

**Magnetic stirrer**

Series 5Q0 from Activon, Australia.

**Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrophotometer**

Voyager DE STR from Applied Biosystems, USA.

**Microplate reader**

Benchmark from BioRad Laboratories, USA.

**pH meter**

Model 250A from Orion Research Inc. (USA) fitted with Activon AEP 433 flat tip probe from Activon Scientific Products Co. Pty. Ltd., Australia. This instrument was calibrated before use according to manufacturer's instructions with standard pH solutions (pH 7.00 and pH 4.01).

**Pipettes**

Gilson Pipetman from John Morris Scientific, Australia. Volumes were calibrated annually by weighing the dispensed volume of dH<sub>2</sub>O at RT to  $\pm 1\%$  of the set volume.

**Platform mixer**

Ratek Model OM6 from Adelab Scientific, Australia.

**Power packs**

Power/Pac Model 300 (for molecular biology studies) and Model 1000 (for proteomic studies) both from BioRad, USA.

**Sequencer**

CEQ<sup>™</sup> 8000 Genetic Analysis System from Beckman Coulter (USA).

**Software and databases**

1. CIA-BEN Version 2.2 from Spiral Biotech (USA) was used for bacterial colony image analysis.
2. Microsoft® Excel 2000 from Microsoft Corporation (USA) was used for data presentation and statistical analysis.
3. UltraFit from Biosoft (UK) was used to generate a predictive model.
4. Kodak 1D Limited Edition 3.5 from Kodak (Australia) was employed to acquire DNA gel images.
5. BioEdit Version 7.0.5 from Ibis Therapeutics (USA) was used to align nucleotide sequences.
6. GLM Version 2.8 from Gemini Data Loggers (UK) was used in the monitoring of temperature.
7. Microplate Manager Version 5.2 from BioRad (USA) was employed to determine protein concentration.
8. STORMScan Version 5.03 from Amersham Biosciences (USA) was used for protein gel image acquisition, which was converted to tiff format using ImageQuant Version 5.2 from Molecular Dynamics (USA).
9. Data Explorer Version 3.5.0.0 from Applied Biosystems (USA) was used to analyse peptide mass data.
10. Mascot from Matrix Science (USA), accessed via <http://www.matrixscience.com>, was used as a search engine to match peptide mass fingerprints with known proteins.
11. UniProt protein knowledge database from ExPASy (Switzerland), accessed via <http://au.expasy.org/>, was used to determine the function of identified proteins.
12. PDQuest 2-D Analysis Version 6.2 from BioRad (USA) was used to analyse protein gels.

**Sonicator**

From Thomas Optical and Scientific, USA.

**Spectrophotometer**

SmartSpec™ 3000 from BioRad, USA.

**Spiral plater**

Autoplate 4000 from Spiral Biotech Inc., USA.

**Spot cutter**

One Touch Plus from The Gel Company, USA.

**Stomacher**

Colworth 400 from AJ Seward, UK.

***Temperature data logger***

Tinytag® from Hastings Data Loggers, Australia.

***Thermocycler***

MJ Research Peltier Thermal Cycler 200 from GeneWorks, Australia.

***UV transilluminator with camera***

UV transilluminator from UVP, USA with an EDAS 290 camera from Kodak, Australia.

***Water activity meter***

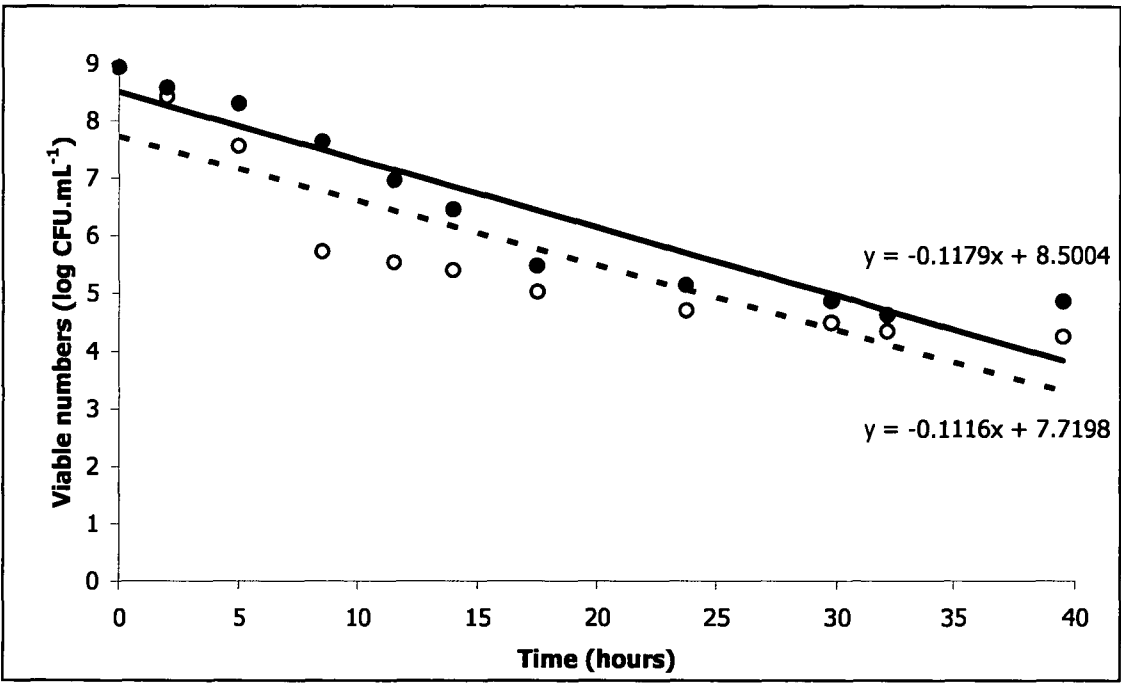
Aqualab CX-2 from Decagon Devices, USA. Calibrated before use according to the manufacturer's instructions.

## APPENDIX B: Modelling *Escherichia coli* Inactivation in Uncooked, Comminuted Fermented Meat using Broth-Based Systems

### B.1 BROTH MODEL DEVELOPMENT

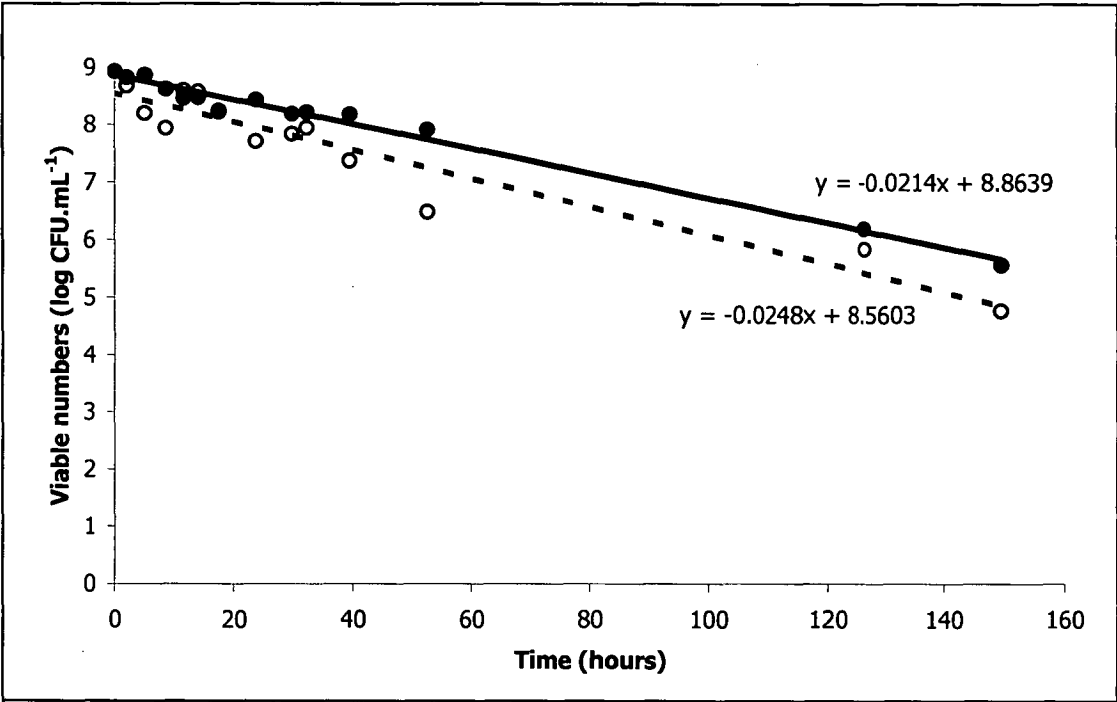
#### B.1.1 Inactivation Kinetics

Depicted in Figures B.1 - B.15 is the inactivation kinetics of *E. coli* in response to combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability (standard or reduced) in CMM- or BHI-based models (Figures B.1 – B.5 and Figures B.6 – B.13 respectively). Assuming log-linear inactivation kinetics, the lines of best fit to the data are shown.

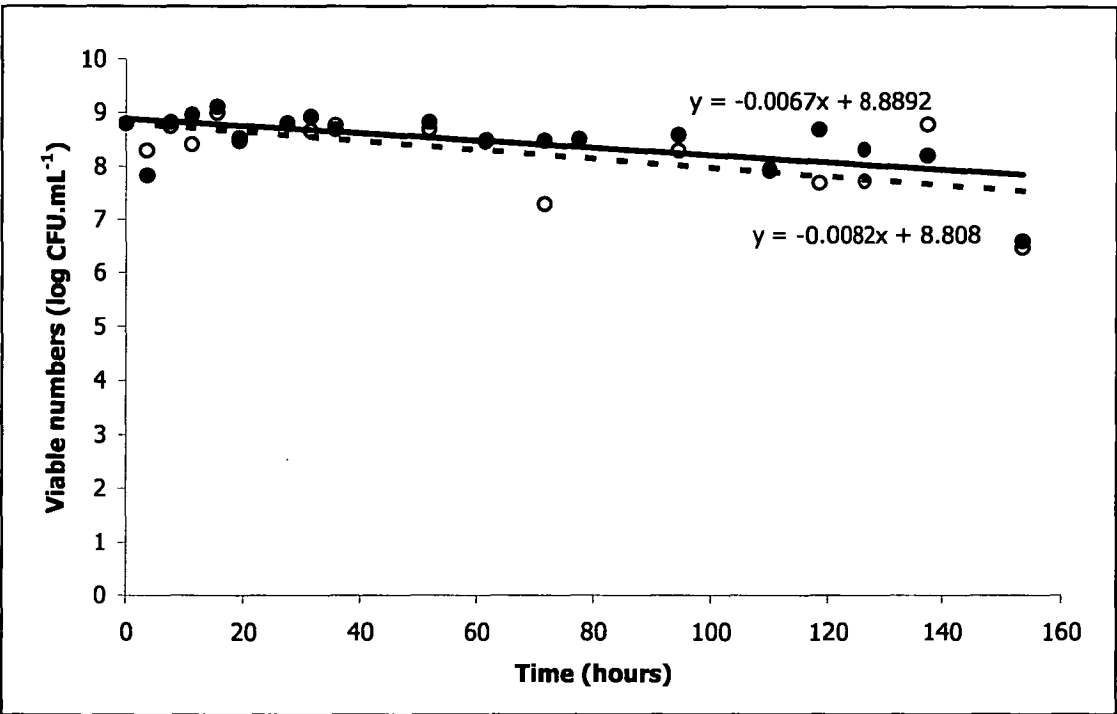


**Figure B.1** Inactivation kinetics of *E. coli* M23 in the CMM-based broth model, at 45°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.947, pH 5.22, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.950, pH 5.66, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

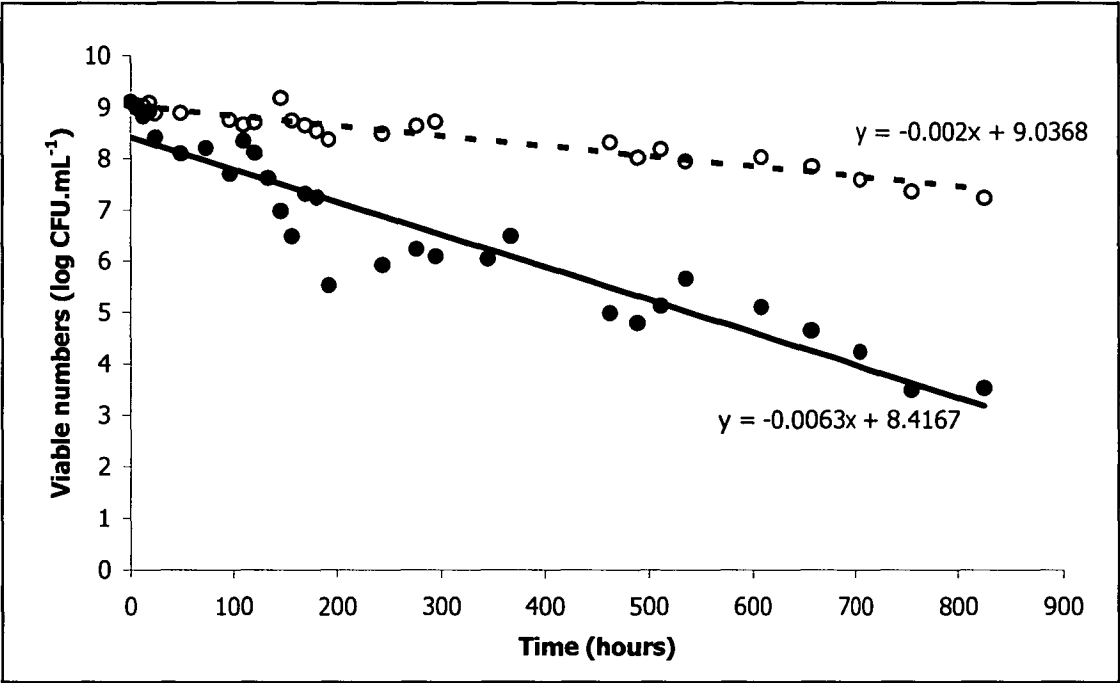




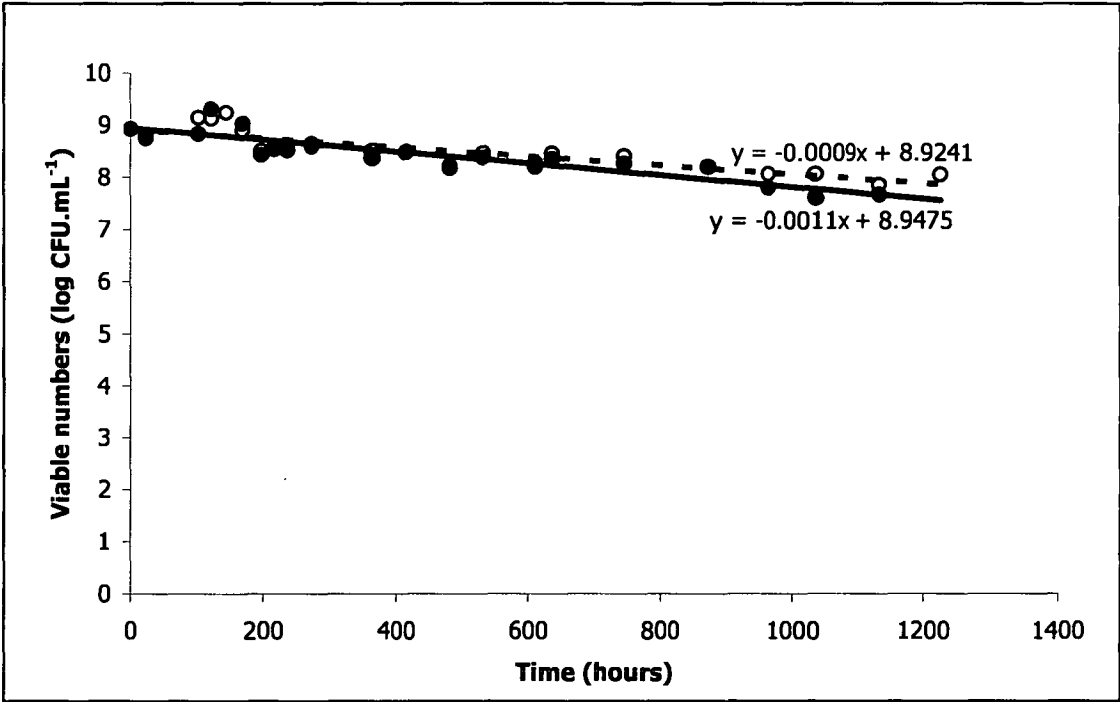
**Figure B.2** Inactivation kinetics of *E. coli* M23 in the CMM-based broth model, at 35°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.945, pH 5.25, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.950, pH 5.74, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



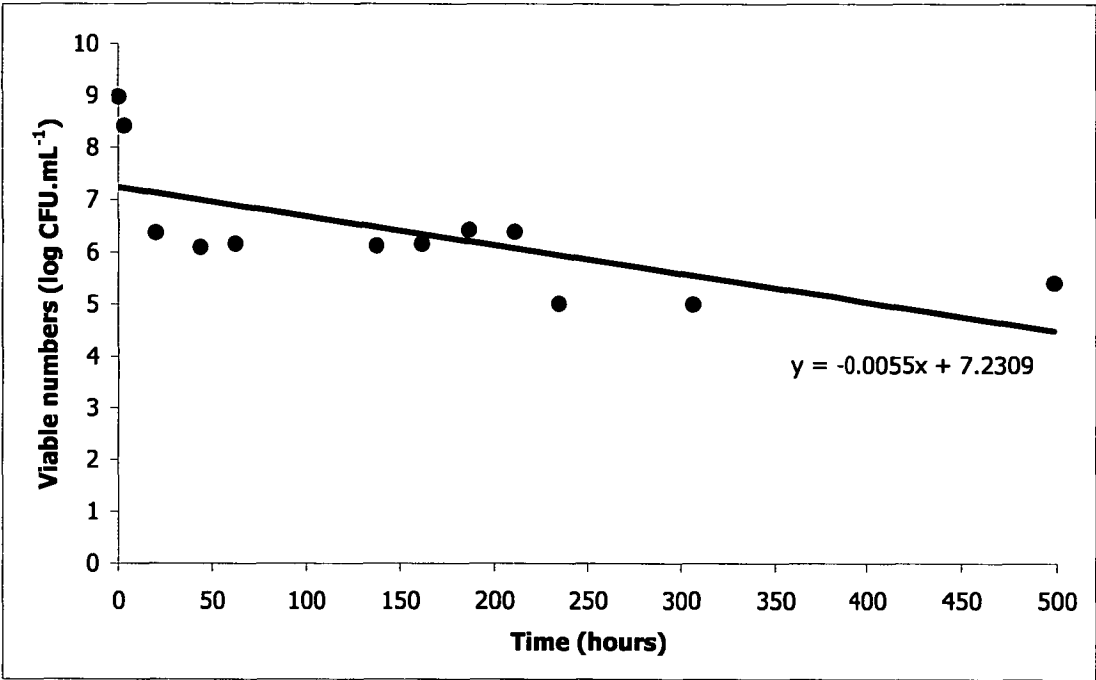
**Figure B.3** Inactivation kinetics of *E. coli* M23 in the CMM-based broth model, at 20°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.954, pH 5.08, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.954, pH 4.98, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



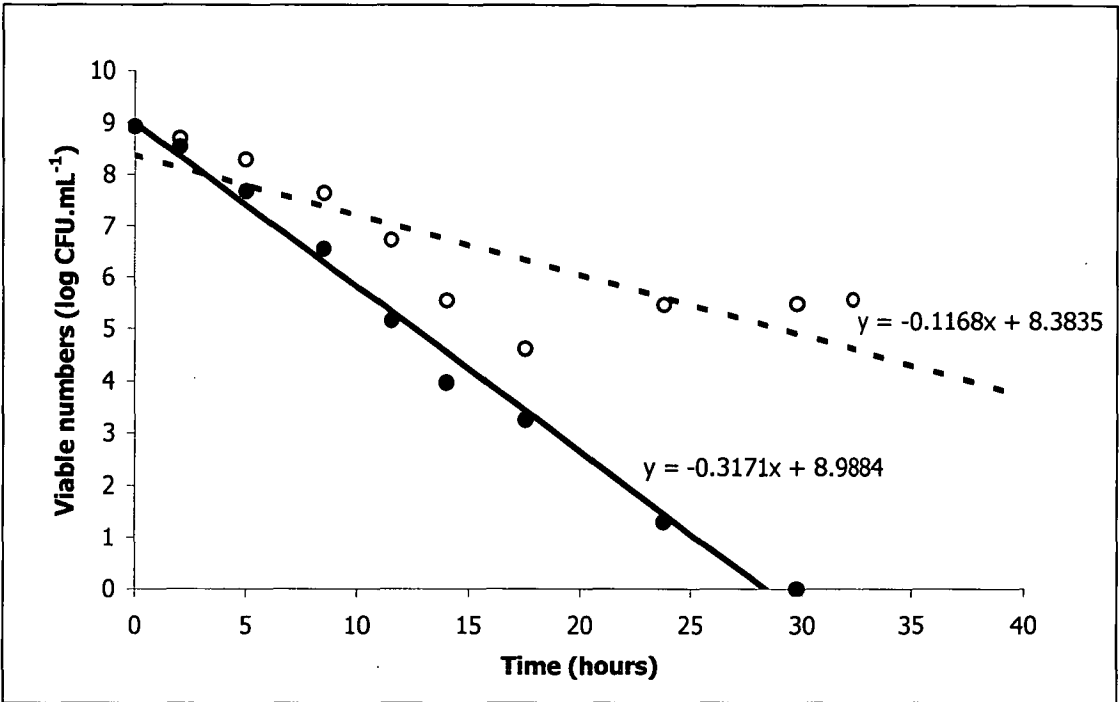
**Figure B.4** Inactivation kinetics of *E. coli* M23 in the CMM-based broth model, at 10°C and with the lines of best fit to the data and equations shown, under standard (●, — ; a<sub>w</sub> 0.879, pH 5.11, 150 mM lactic acid) or reduced (○, ---; a<sub>w</sub> 0.959, pH 4.33, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



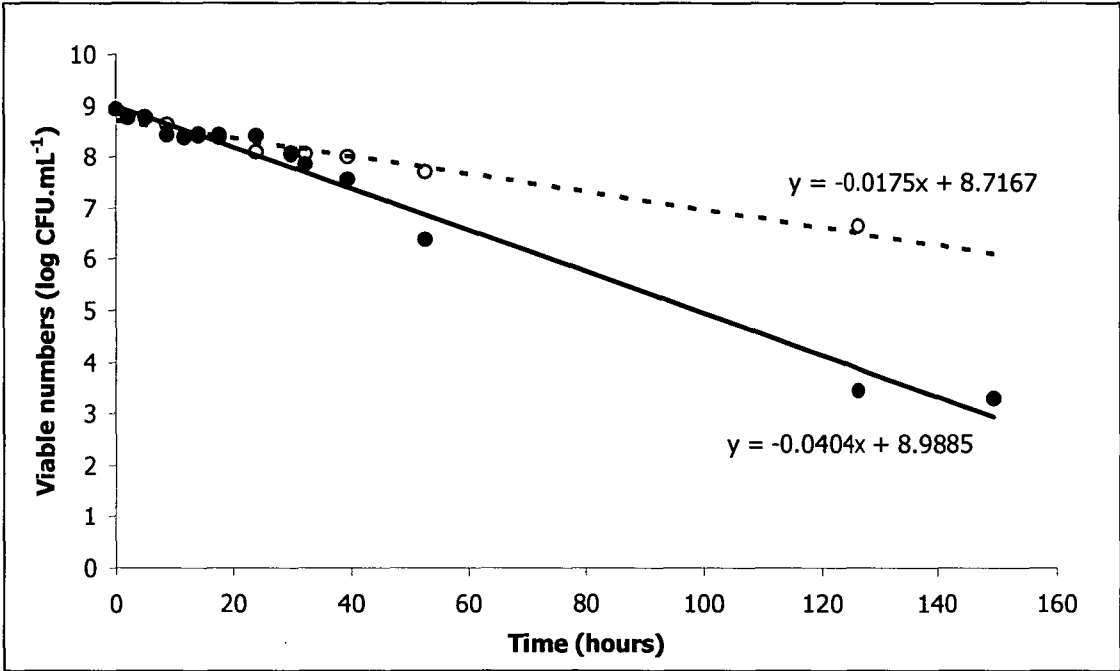
**Figure B.5** Inactivation kinetics of *E. coli* M23 in the CMM-based broth model, at 5°C, under reduced oxygen conditions and with the lines of best fit to the data and equations shown (●, — ; a<sub>w</sub> 0.967, pH 5.03, 150 mM lactic acid; ○, ---; a<sub>w</sub> 0.964, pH 4.99, 150 mM lactic acid). Viable cells were enumerated on BHA-P at 37°C for 14 hours.



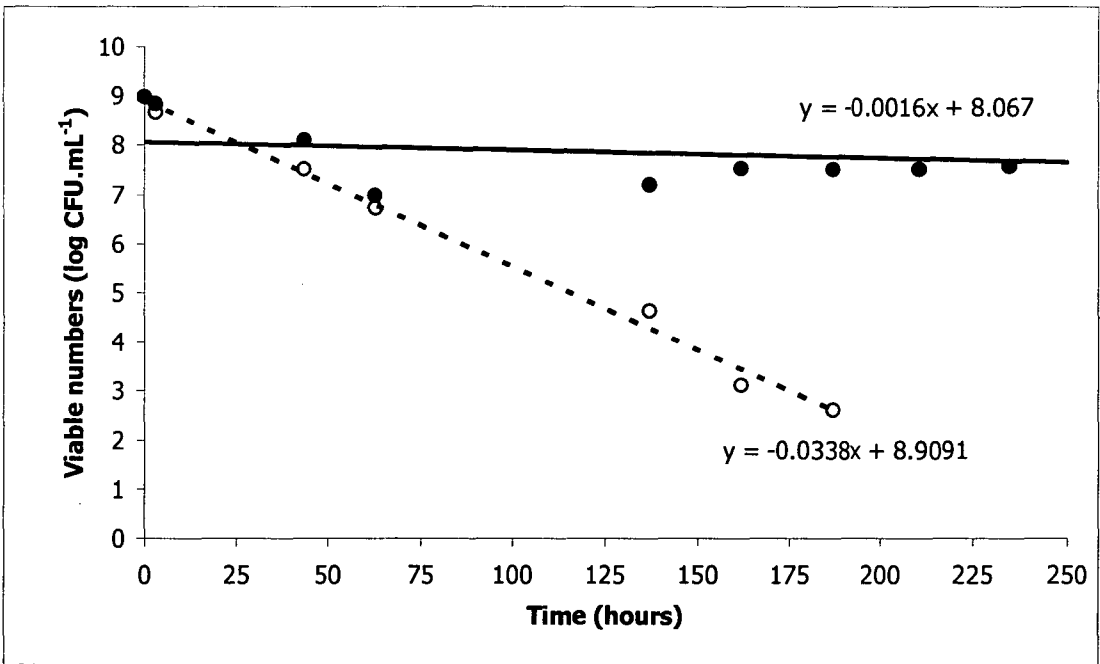
**Figure B.6** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 45°C, under standard oxygen conditions ( $a_w$  0.950, pH 5.00, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



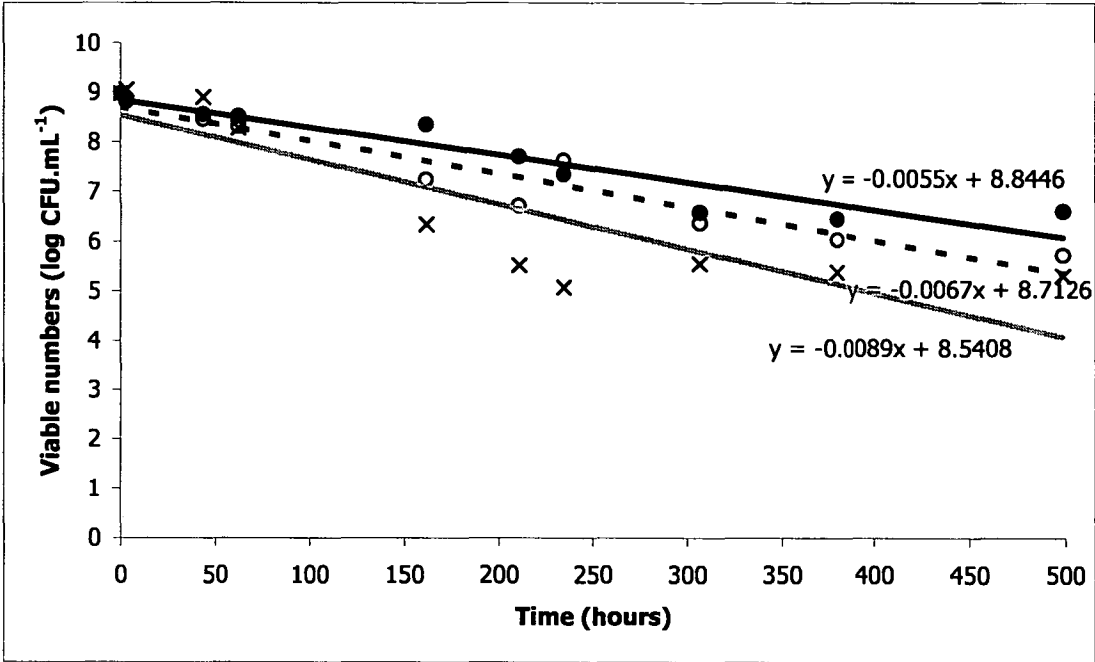
**Figure B.7** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 45°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.941, pH 4.91, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.932, pH 4.84, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



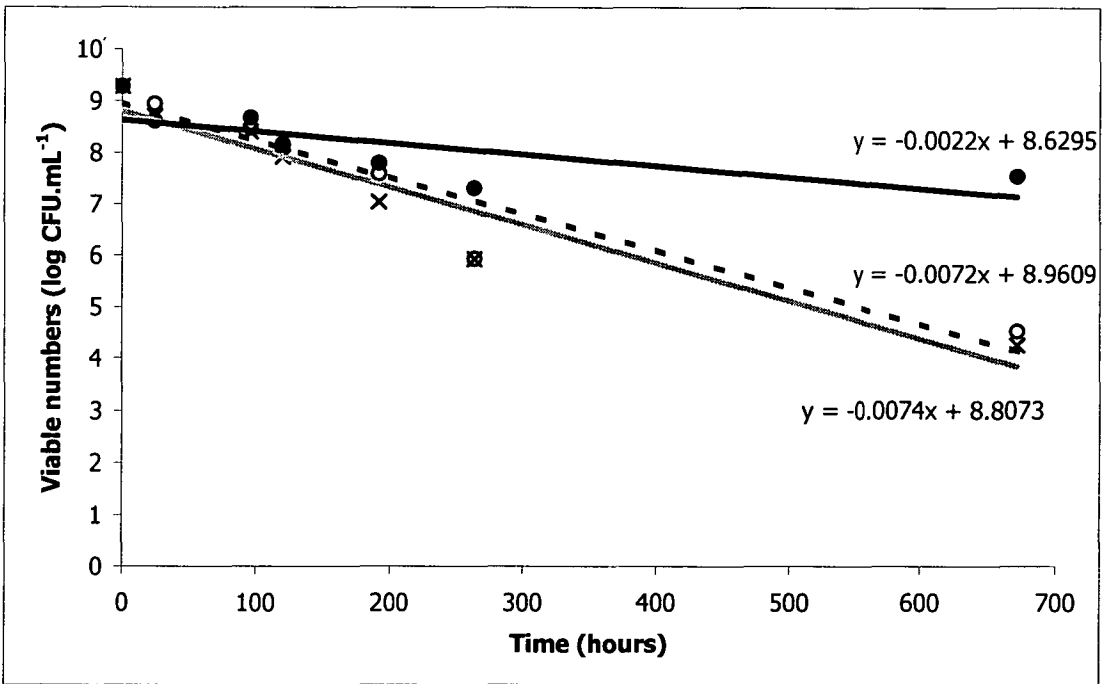
**Figure B.8** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 35°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.940, pH 4.98, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.940, pH 4.92, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



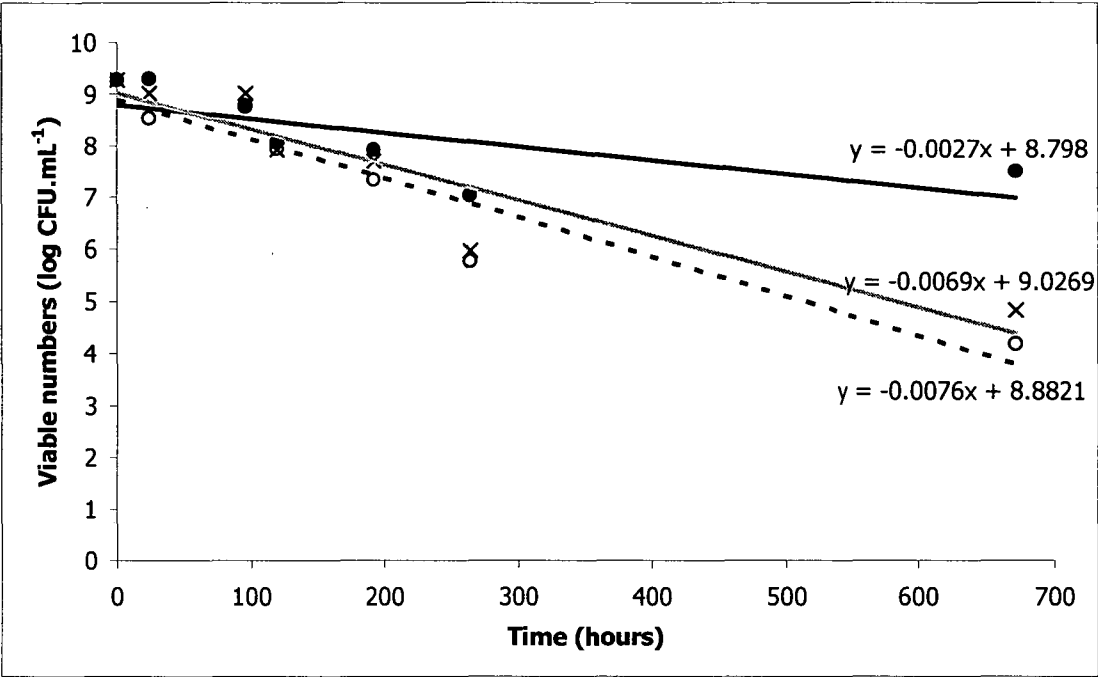
**Figure B.9** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 35°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.951, pH 4.98, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.948, pH 4.94, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



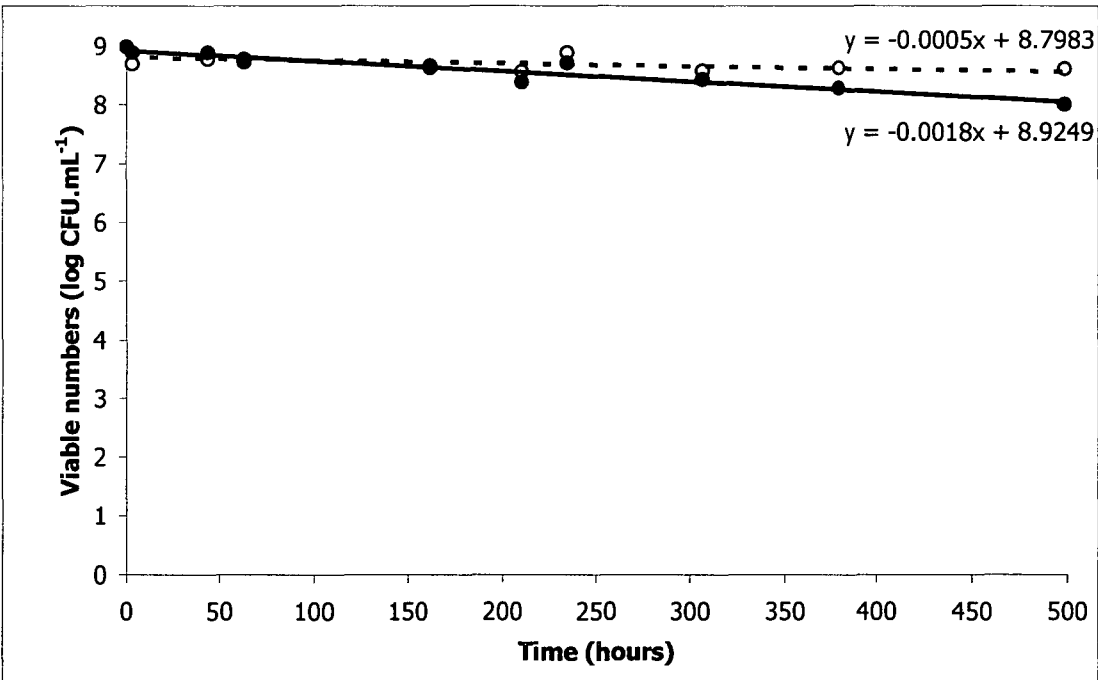
**Figure B.10** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 25°C and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.954, pH 4.92, 150 mM lactic acid; x, ---;  $a_w$  0.949, pH 5.01, without lactic acid) or reduced (○, ---;  $a_w$  0.949, pH 4.98, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



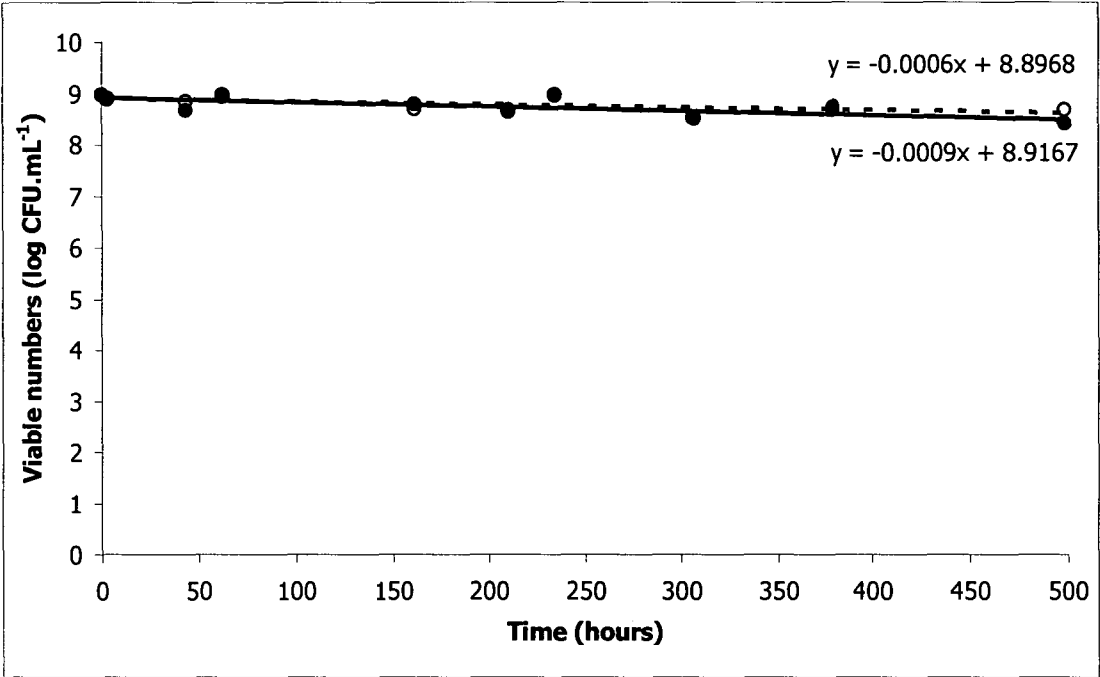
**Figure B.11** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 25°C and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.949, pH 5.11, without lactic acid; and x, ---;  $a_w$  0.953, pH 4.91, without lactic acid, without shaking of the flask) or reduced (○, ---;  $a_w$  0.941, pH 4.90, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



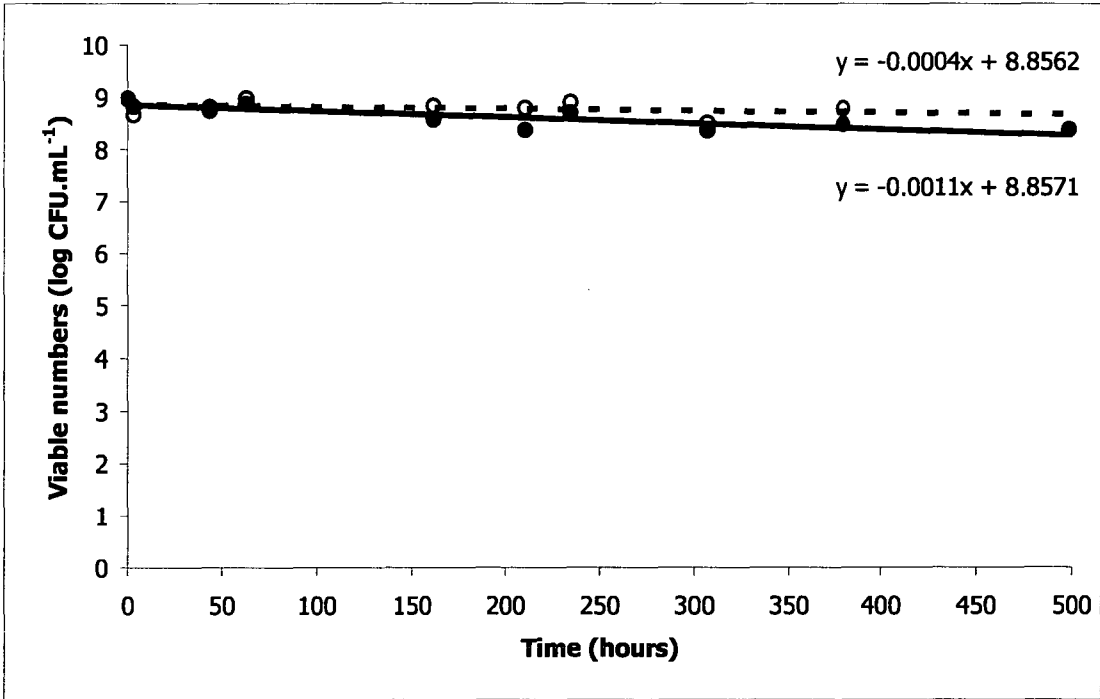
**Figure B.12** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 25°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.961, pH 5.53, without lactic acid; and x, —;  $a_w$  0.955, pH 5.21, without lactic acid, without shaking of the flask) or reduced (○, ---;  $a_w$  0.943, pH 5.15, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure B.13** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 15°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.952, pH 5.01, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.944, pH 5.08, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure B.14** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 5°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.950, pH 4.81, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.954, pH 4.65, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure B.15** Inactivation kinetics of *E. coli* M23 in the BHI-based broth model, at 5°C and with the lines of best fit to the data and equations shown, under standard (●, — ;  $a_w$  0.955, pH 5.21, without lactic acid) or reduced (○, ---;  $a_w$  0.948, pH 4.95, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

### B.1.2 Combination of Conditions Tested and the Observed Rates of Inactivation

The inactivation rates of *E. coli* in response to combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability (standard or reduced) in CMM- or BHI-based broth models was calculated from the lines of best fit to the data shown in Appendix B.1.1. This set of 32 inactivation rate data is shown in Table B.1.

**Table B.1** The combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability tested in CMM- or BHI-based models and the overall inactivation rates of *E. coli* calculated from the line of best fit to the data shown in Appendix B.1.1.

| Broth type | Temp. (°C) | $a_w$ | pH   | Lactic acid conc. (mM) | Oxygen availability | Inactivation rate <sup>a</sup> (log CFU.mL <sup>-1</sup> .hr <sup>-1</sup> ) | see Figure |
|------------|------------|-------|------|------------------------|---------------------|--|------------|
| CMM        | 45         | 0.950 | 5.66 | 150                    | Reduced             | 0.1116   | B1         |
|            | 45         | 0.947 | 5.22 | 150                    | Standard            | 0.1179   | B1         |
|            | 35         | 0.950 | 5.74 | 150                    | Reduced             | 0.0248   | B2         |
|            | 35         | 0.945 | 5.25 | 150                    | Standard            | 0.0214   | B2         |
|            | 20         | 0.954 | 5.08 | 150                    | Standard            | 0.0067   | B3         |
|            | 20         | 0.954 | 4.98 | 150                    | Reduced             | 0.0082   | B3         |
|            | 10         | 0.959 | 5.33 | 150                    | Reduced             | 0.0020   | B4         |
|            | 10         | 0.879 | 5.11 | 150                    | Standard            | 0.0063   | B4         |
|            | 5          | 0.967 | 5.03 | 150                    | Reduced             | 0.0011   | B5         |
|            | 5          | 0.964 | 4.99 | 150                    | Reduced             | 0.00090  | B5         |
| BHI        | 45         | 0.950 | 5.00 | 150                    | Standard            | 0.0055   | B6         |
|            | 45         | 0.941 | 4.91 | 150                    | Standard            | 0.3171   | B7         |
|            | 45         | 0.932 | 4.84 | 150                    | Reduced             | 0.1168   | B7         |
|            | 35         | 0.940 | 4.98 | 150                    | Standard            | 0.0404   | B8         |
|            | 35         | 0.940 | 4.92 | 150                    | Reduced             | 0.0175   | B8         |
|            | 35         | 0.951 | 4.98 | 150                    | Standard            | 0.0016   | B9         |



(Table B.1 continued ...)

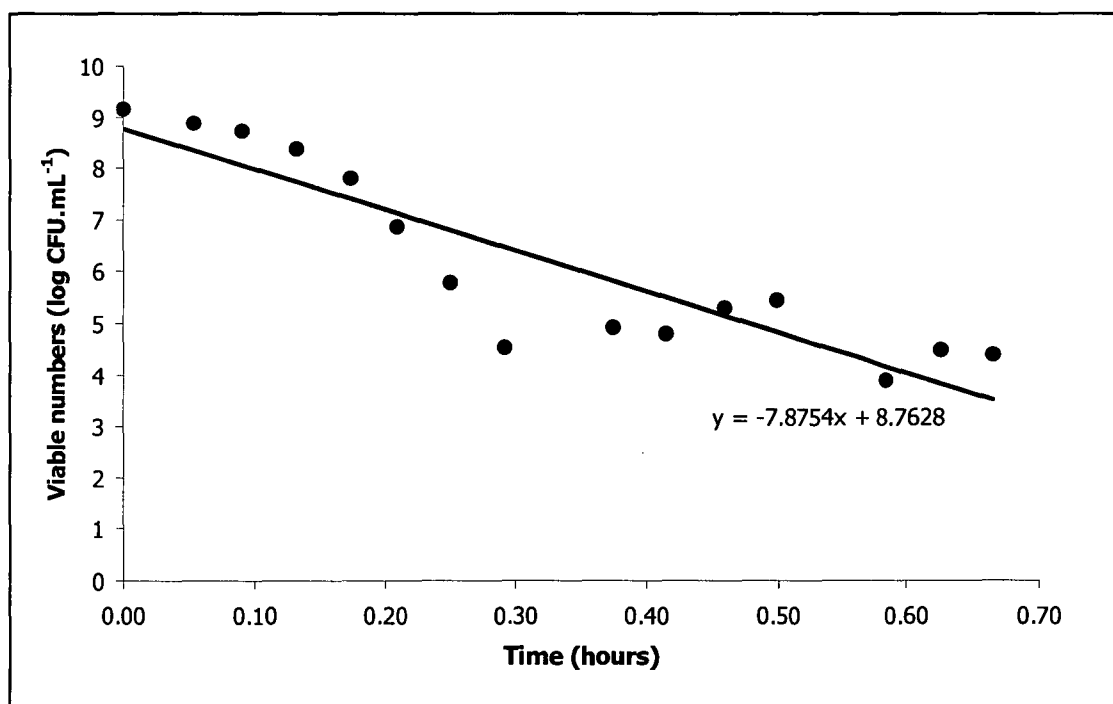
| Broth type | Temp. (°C) | a <sub>w</sub> | pH   | Lactic acid conc. (mM) | Oxygen availability | Inactivation rate <sup>a</sup> (log CFU.mL <sup>-1</sup> .hr <sup>-1</sup> ) | see Figure |
|------------|------------|----------------|------|------------------------|---------------------|--|------------|
| BHI        | 35         | 0.948          | 4.94 | 150                    | Reduced             | 0.0338   | B9         |
|            | 25         | 0.954          | 4.92 | 150                    | Standard            | 0.0055   | B10        |
|            | 25         | 0.949          | 5.01 | 0                      | Standard            | 0.0089   | B10        |
|            | 25         | 0.949          | 4.98 | 150                    | Reduced             | 0.0067   | B10        |
|            | 25         | 0.953          | 4.91 | 0                      | Standard            | 0.0074   | B11        |
|            | 25         | 0.955          | 5.21 | 0                      | Standard            | 0.0069   | B12        |
|            | 25         | 0.969          | 5.53 | 0                      | Standard            | 0.0027   | B12        |
|            | 25         | 0.949          | 5.11 | 0                      | Standard            | 0.0022   | B11        |
|            | 25         | 0.941          | 4.90 | 0                      | Reduced             | 0.0072   | B11        |
|            | 25         | 0.943          | 5.15 | 0                      | Reduced             | 0.0076   | B12        |
|            | 15         | 0.952          | 5.01 | 150                    | Standard            | 0.0018   | B13        |
|            | 15         | 0.944          | 5.08 | 150                    | Reduced             | 0.0005   | B13        |
|            | 5          | 0.950          | 4.81 | 150                    | Standard            | 0.0009   | B14        |
|            | 5          | 0.955          | 5.21 | 0                      | Standard            | 0.0011   | B15        |
|            | 5          | 0.954          | 4.65 | 150                    | Reduced             | 0.0006   | B14        |
|            | 5          | 0.948          | 4.95 | 0                      | Reduced             | 0.0004   | B15        |

<sup>a</sup> Overall inactivation rate based on the line of best fit to the data assuming log-linear inactivation.

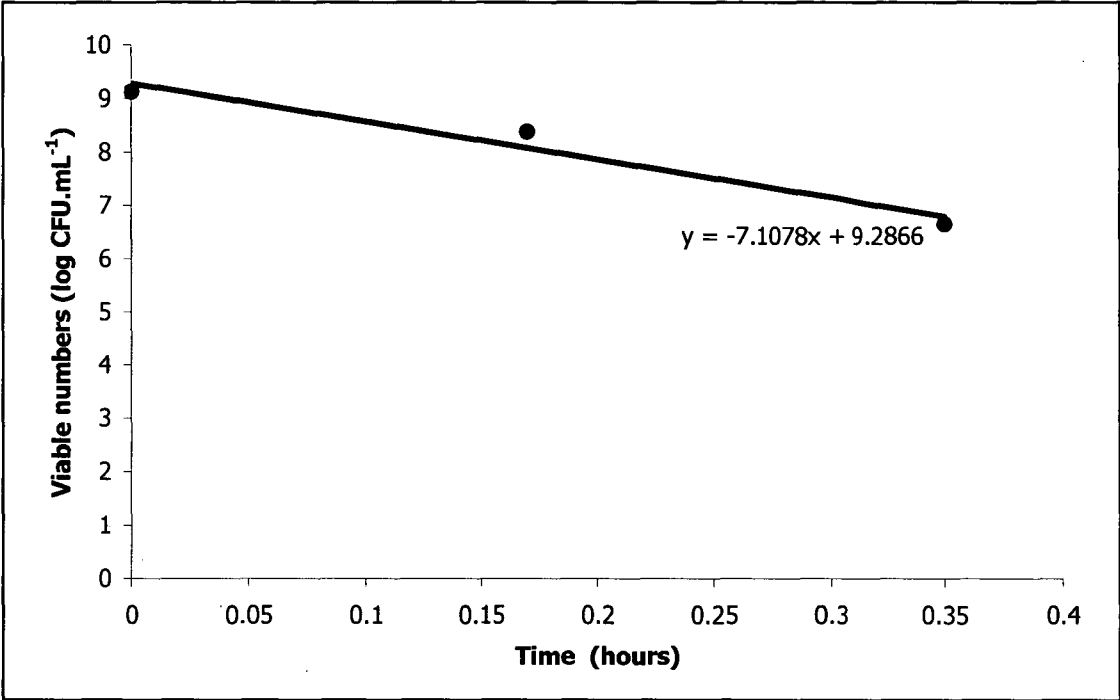
## B.2 DETERMINATION OF INACTIVATION RATES USING THE BROTH MODEL

### B.2.1 Inactivation Kinetics of *Escherichia coli*

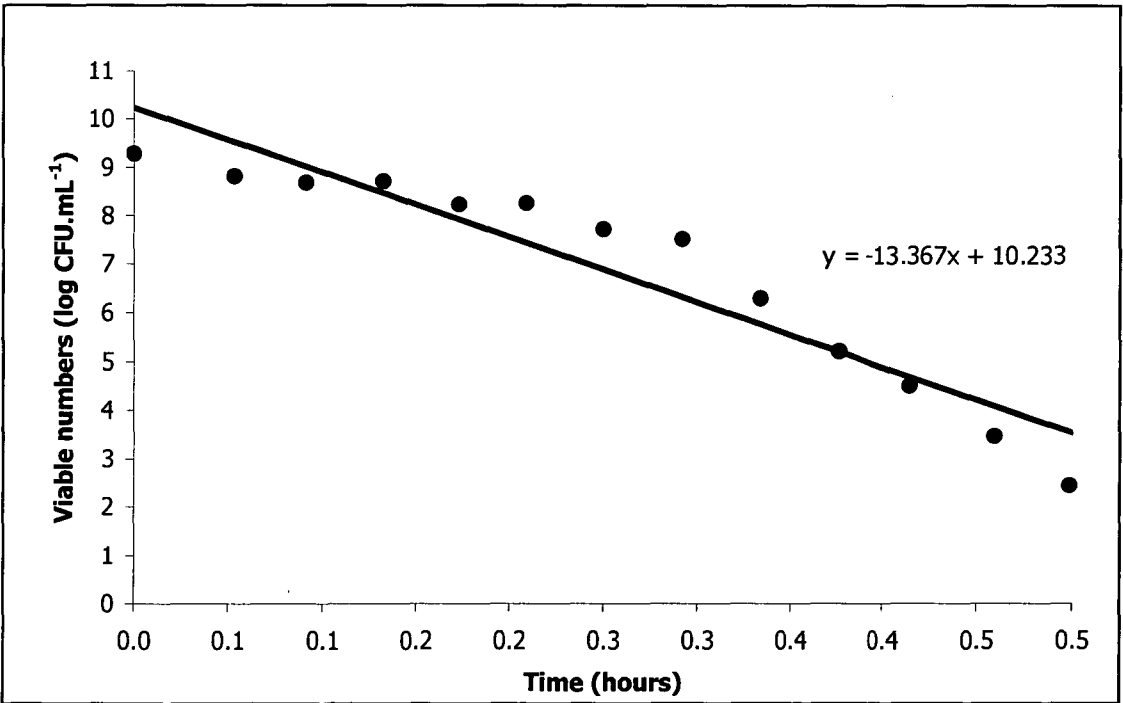
Using the broth model developed, the inactivation kinetics of *E. coli* in response to combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability (standard or reduced) were determined and are shown in Figures B.16 - B.55, which includes CMM-based broth model data previously described in Appendix B.1. Assuming log-linear inactivation kinetics, the lines of best fit to the data are shown.



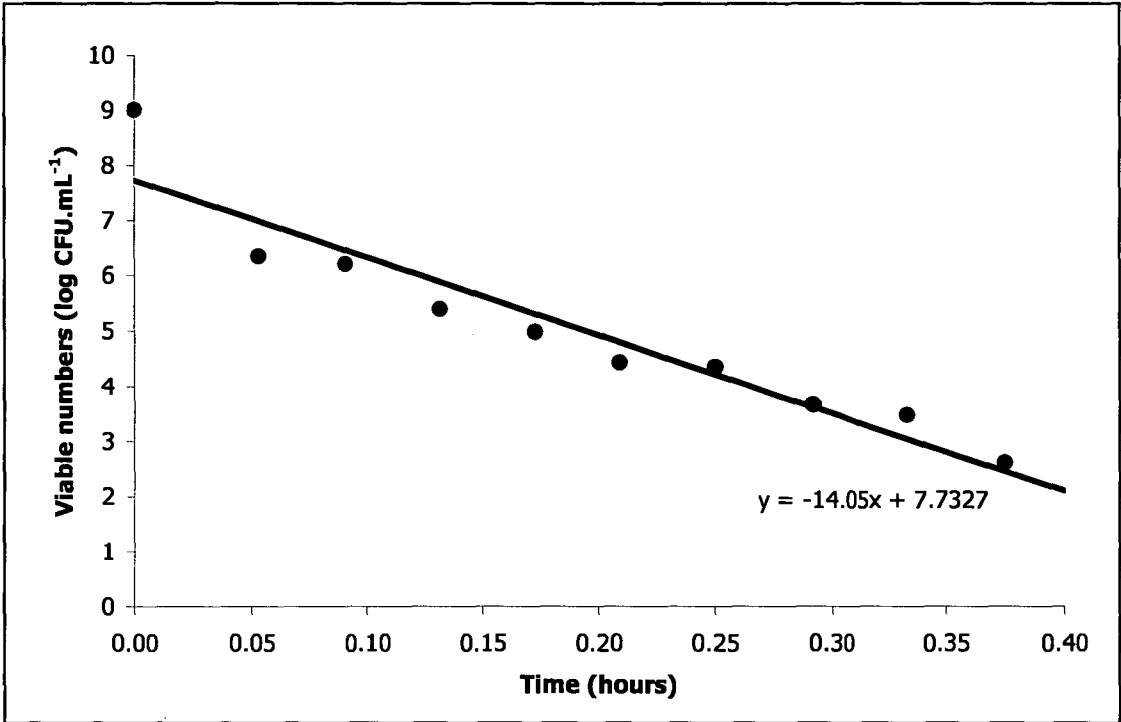
**Figure B.16** Inactivation kinetics of *E. coli* M23 in the broth model at 55°C, with  $a_w$  0.97 under standard oxygen conditions ( $a_w$  0.971, pH 4.14, lactic acid 150 Mm). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



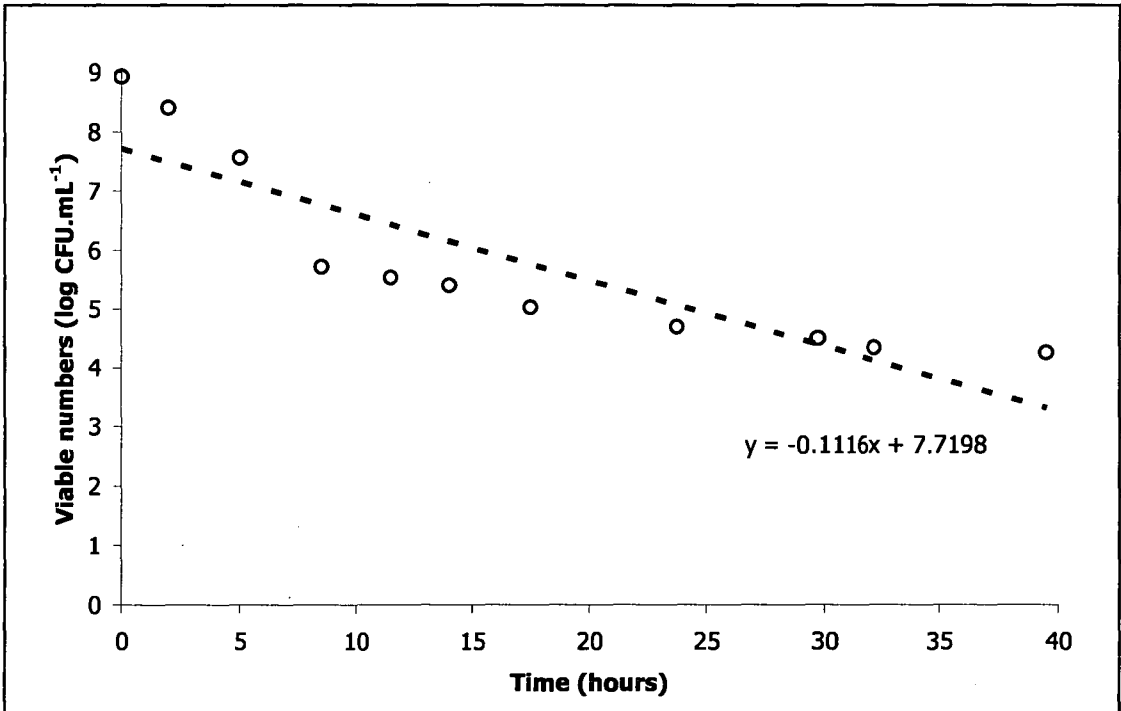
**Figure B.17** Inactivation kinetics of *E. coli* M23 in the broth model at 55°C, with  $a_w$  0.96 under standard oxygen conditions ( $a_w$  0.962, pH 4.55, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



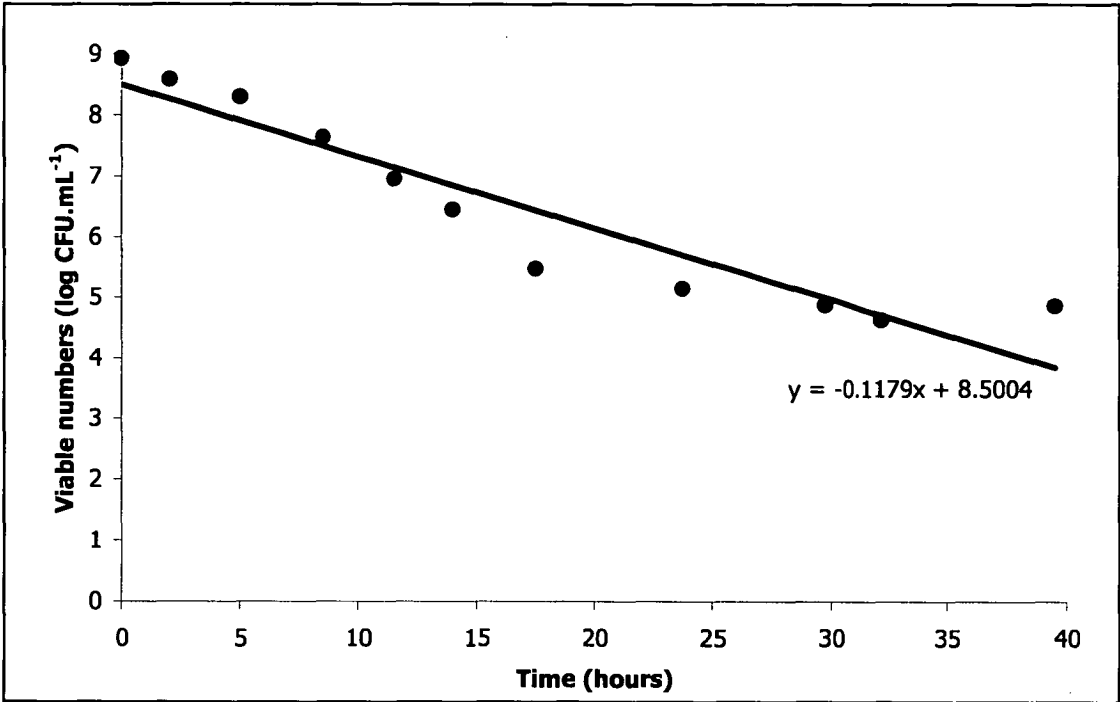
**Figure B.18** Inactivation kinetics of *E. coli* M23 in the broth model at 55°C, with  $a_w$  0.92 under standard oxygen conditions ( $a_w$  0.929, pH 4.90, 150mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



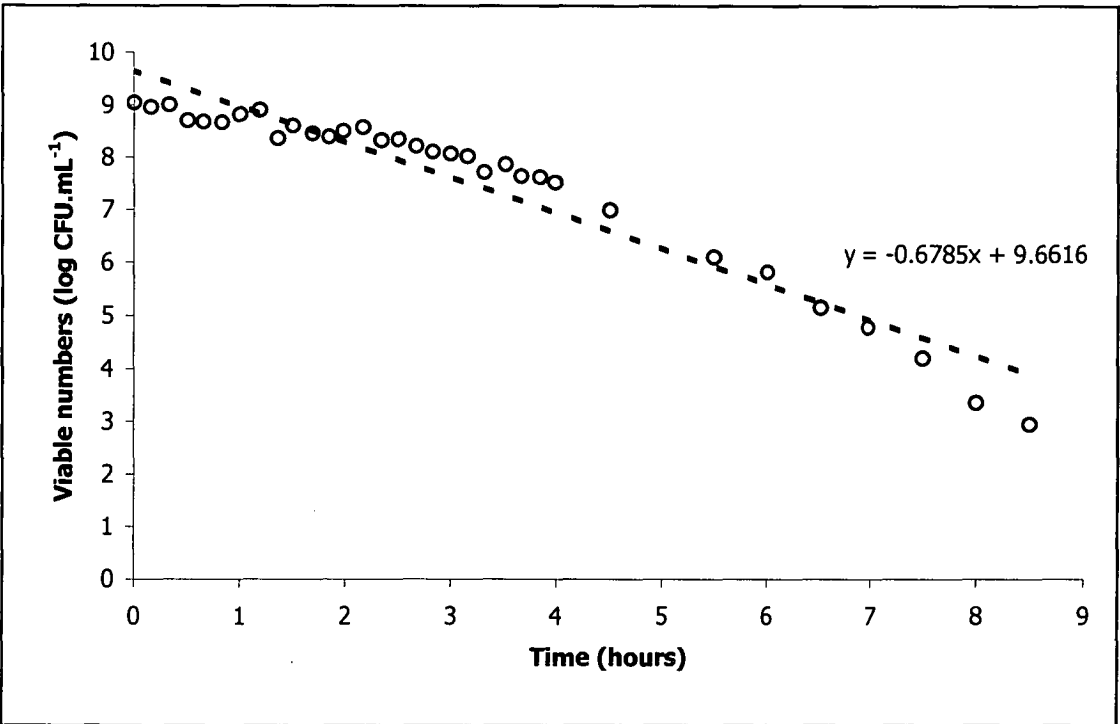
**Figure B.19** Inactivation kinetics of *E. coli* M23 in the broth model at 55°C, with  $a_w$  0.87 under standard oxygen conditions ( $a_w$  0.873, pH 4.71, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



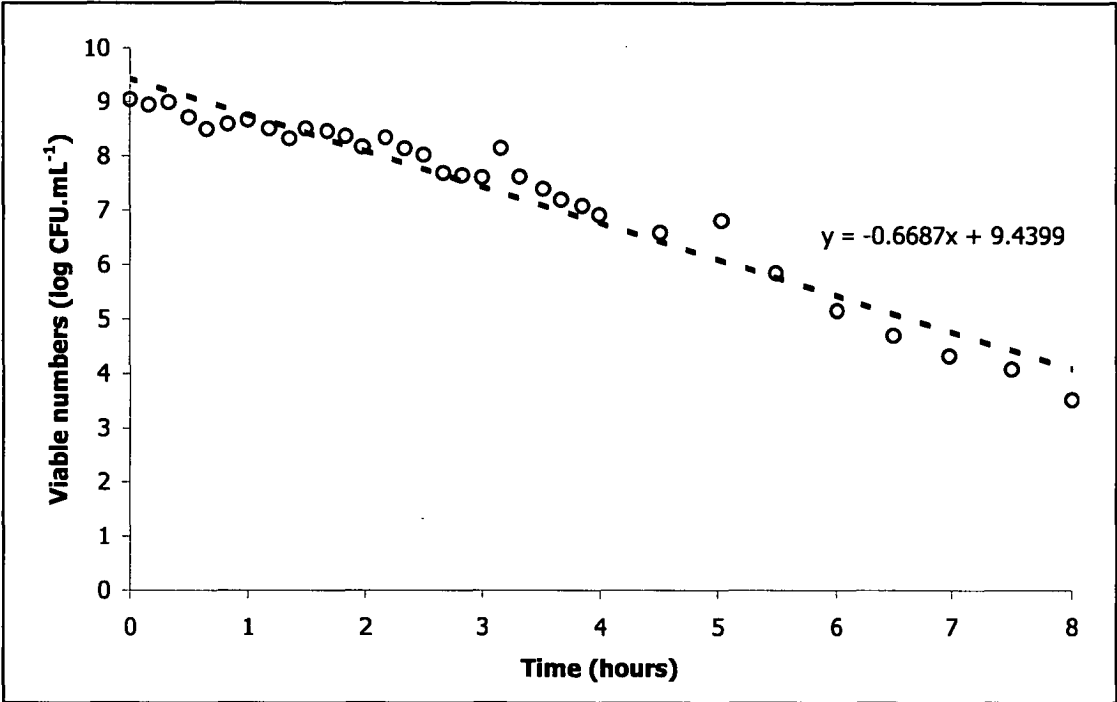
**Figure B.20** Inactivation kinetics of *E. coli* M23 in the broth model at 45°C, with  $a_w$  0.95 under reduced oxygen conditions ( $a_w$  0.950, pH 5.66, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.1.



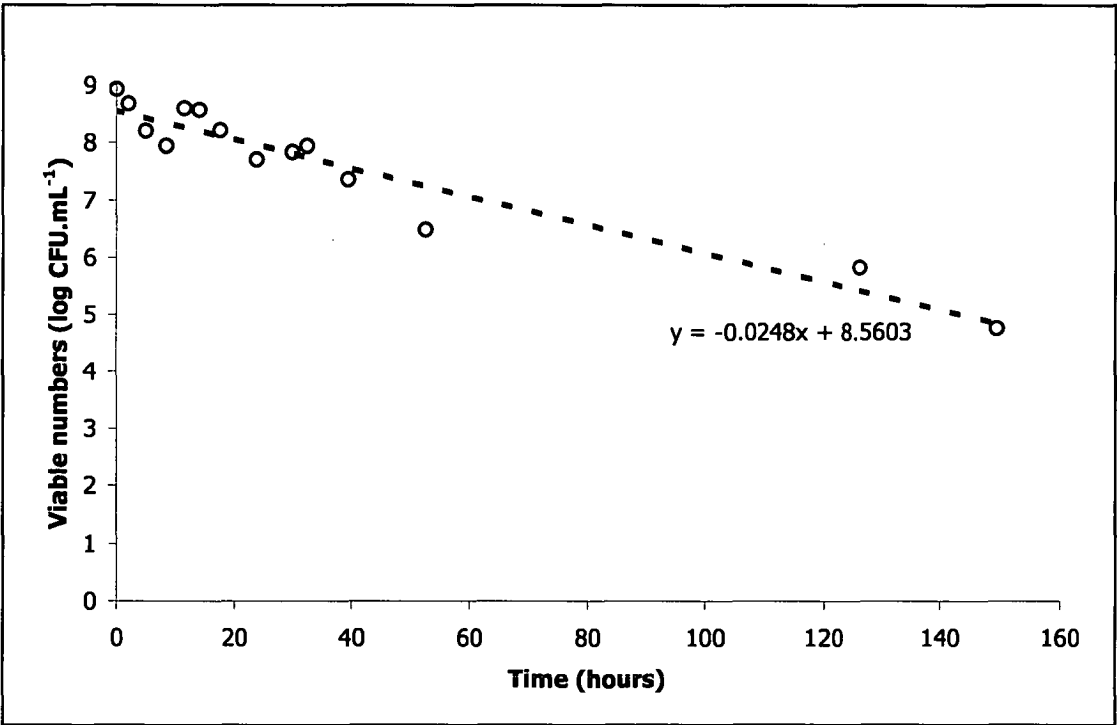
**Figure B.21** Inactivation kinetics of *E. coli* M23 in the broth model at 45°C, with  $a_w$  0.94 under standard oxygen conditions ( $a_w$  0.947, pH 5.22, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.1.



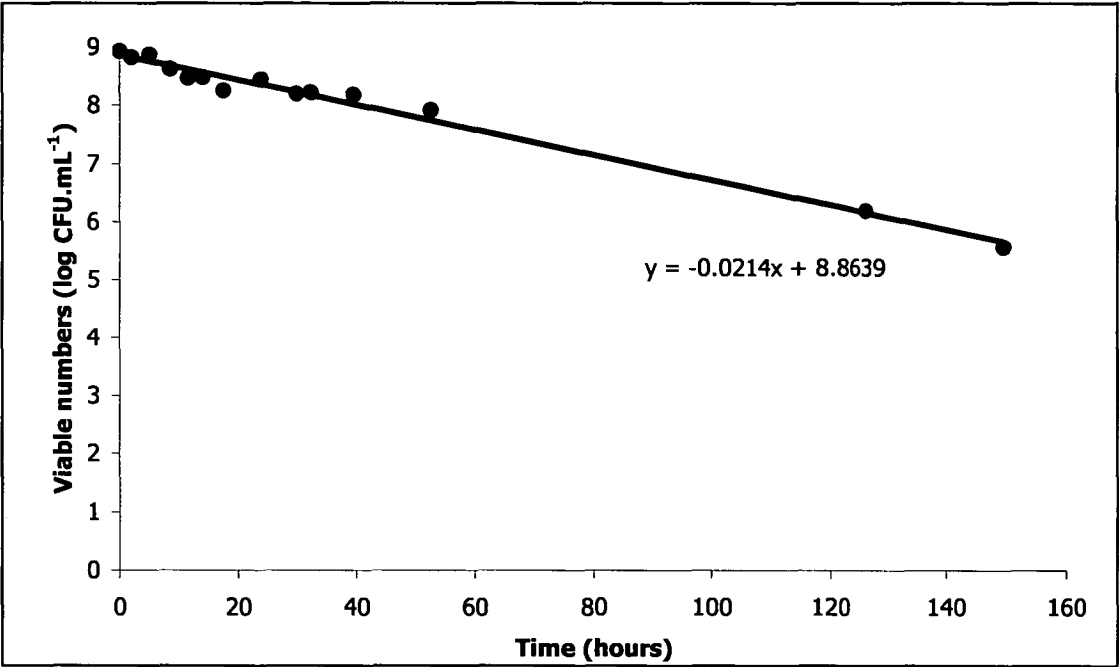
**Figure B.22** Inactivation kinetics of *E. coli* M23 in the broth model at 45°C, with  $a_w$  0.92 under reduced oxygen conditions ( $a_w$  0.928, pH 4.97, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



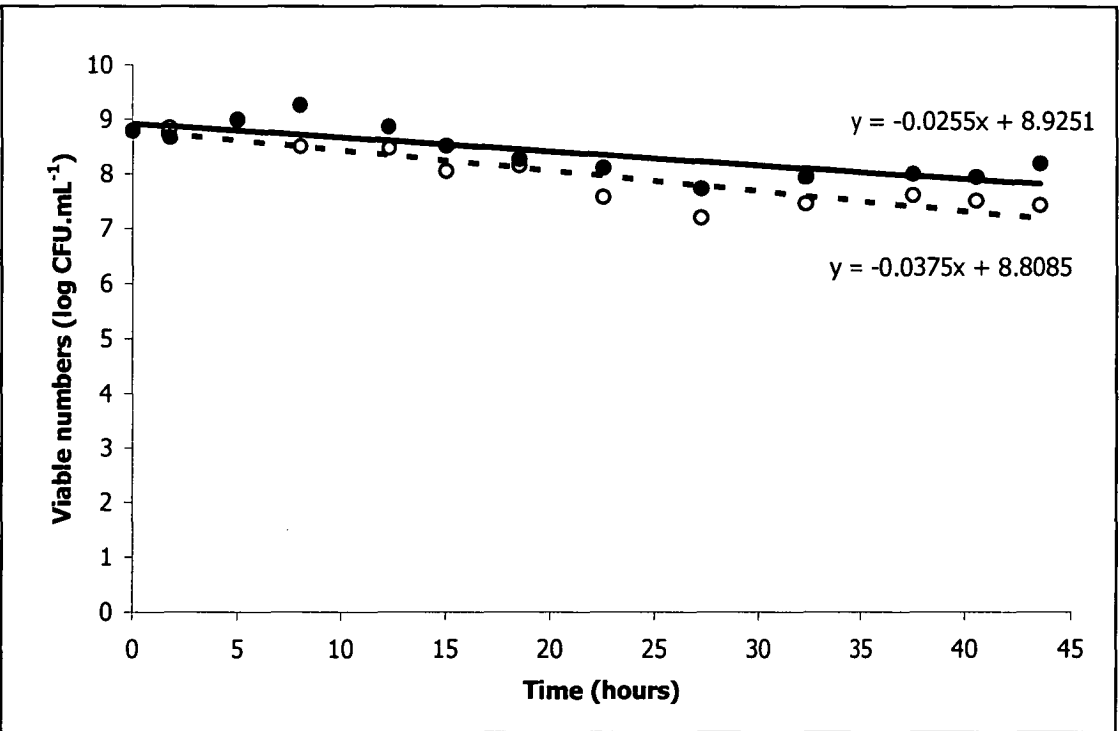
**Figure B.23** Inactivation kinetics of *E. coli* M23 in the broth model at 45°C, with  $a_w$  0.89 under reduced oxygen conditions ( $a_w$  0.895, pH 5.10, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



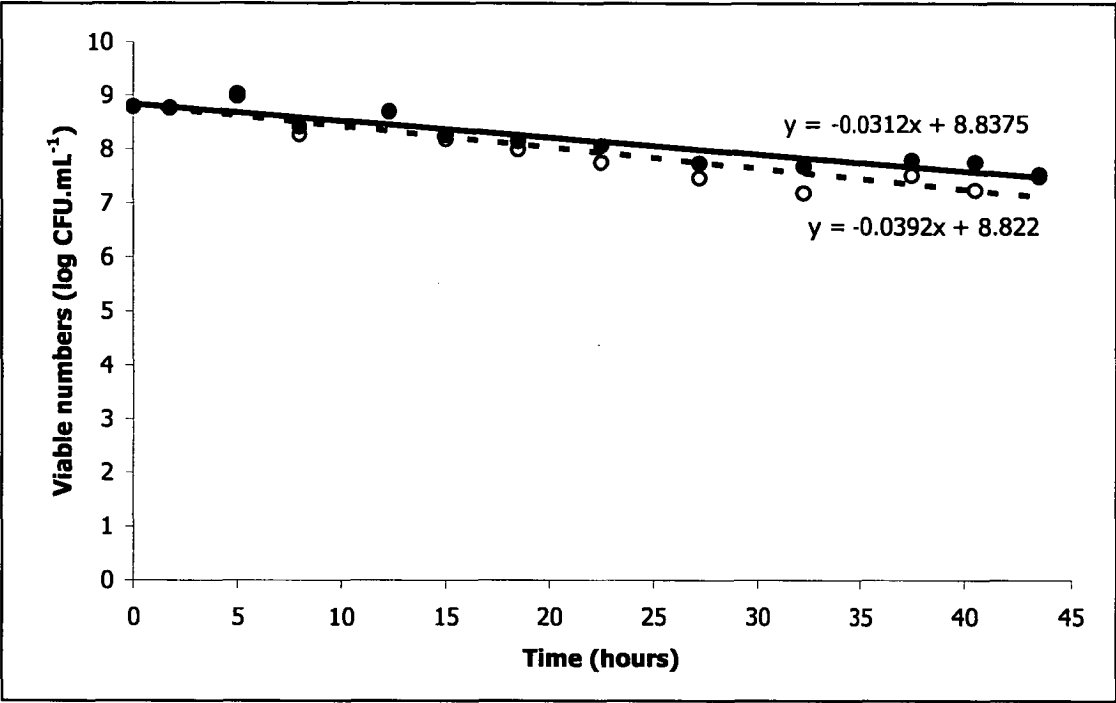
**Figure B.24** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.95 under reduced oxygen conditions ( $a_w$  0.950, pH 5.74, lactic acid 150 mM). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.2.



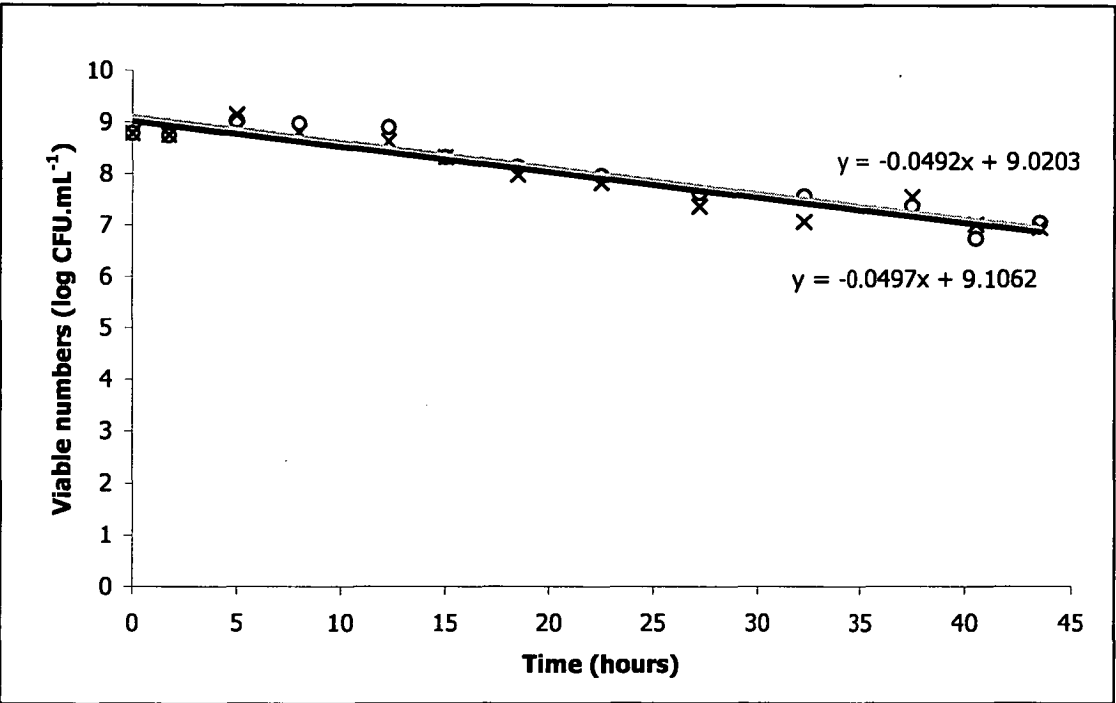
**Figure B.25** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.94 under standard oxygen conditions ( $a_w$  0.945, pH 5.25, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.2.



**Figure B.26** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.94 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.941, pH 6.15, without lactic acid) or reduced (○, ---;  $a_w$  0.944, pH 5.94, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

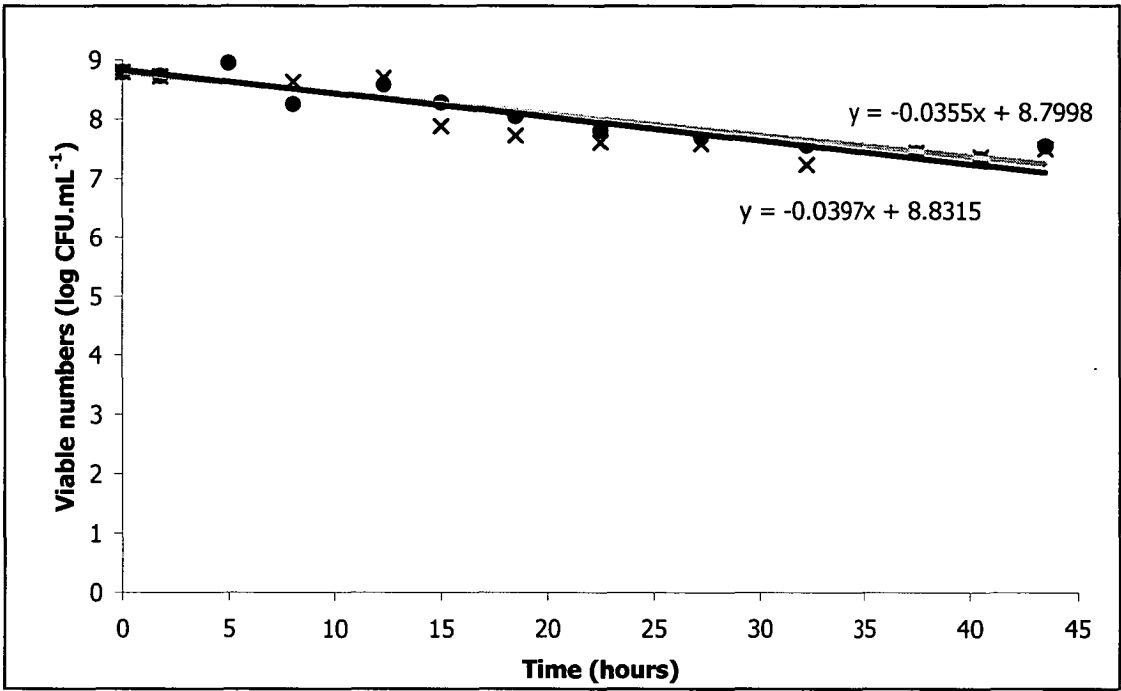


**Figure B.27** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.94 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.940, pH 6.13, without lactic acid) or reduced (○, ---;  $a_w$  0.943, pH 5.84, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

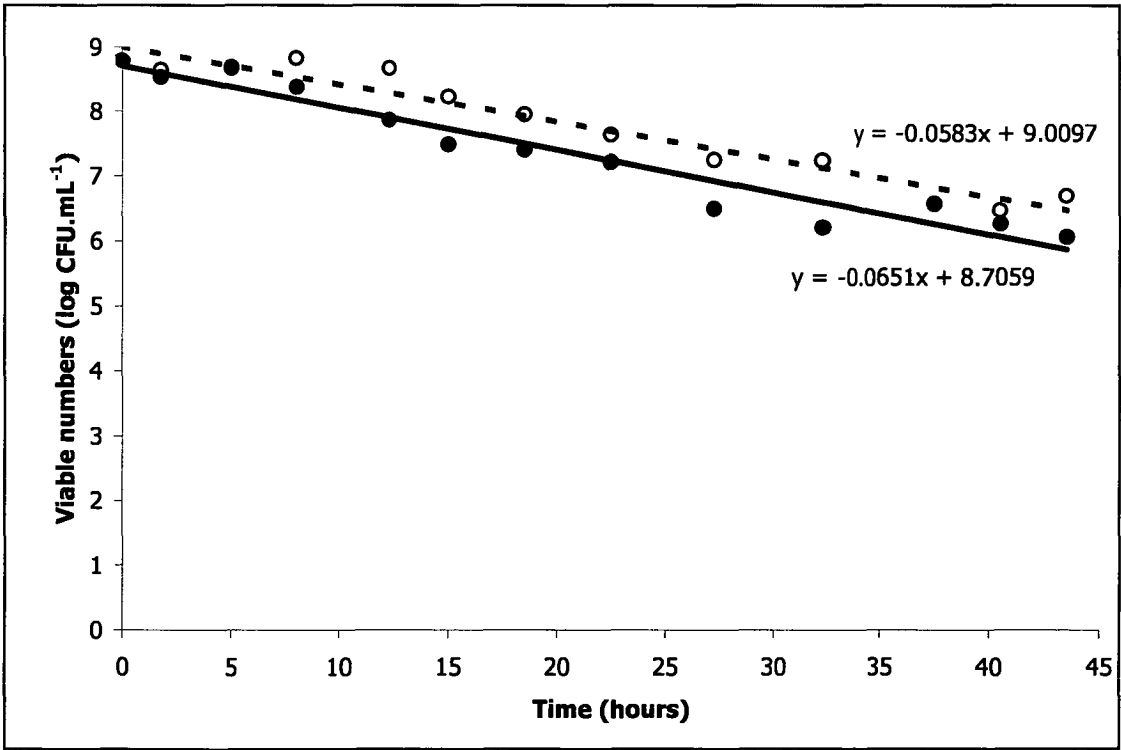


**Figure B.28** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.93 and with the lines of best fit to the data and equations shown, under reduced oxygen conditions (○, —;  $a_w$  0.932, pH 5.74, without lactic acid; or ×, —;  $a_w$  0.930, pH 5.57, without lactic acid). Viable cells were enumerated on BHA-P at 37°C for 14 hours.

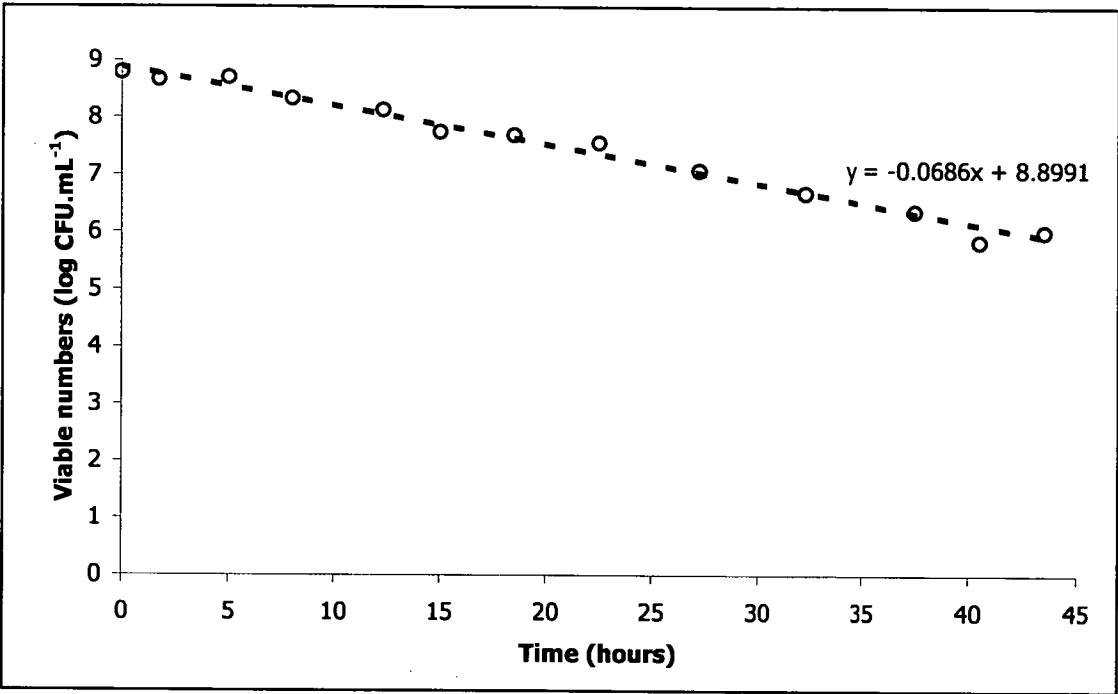




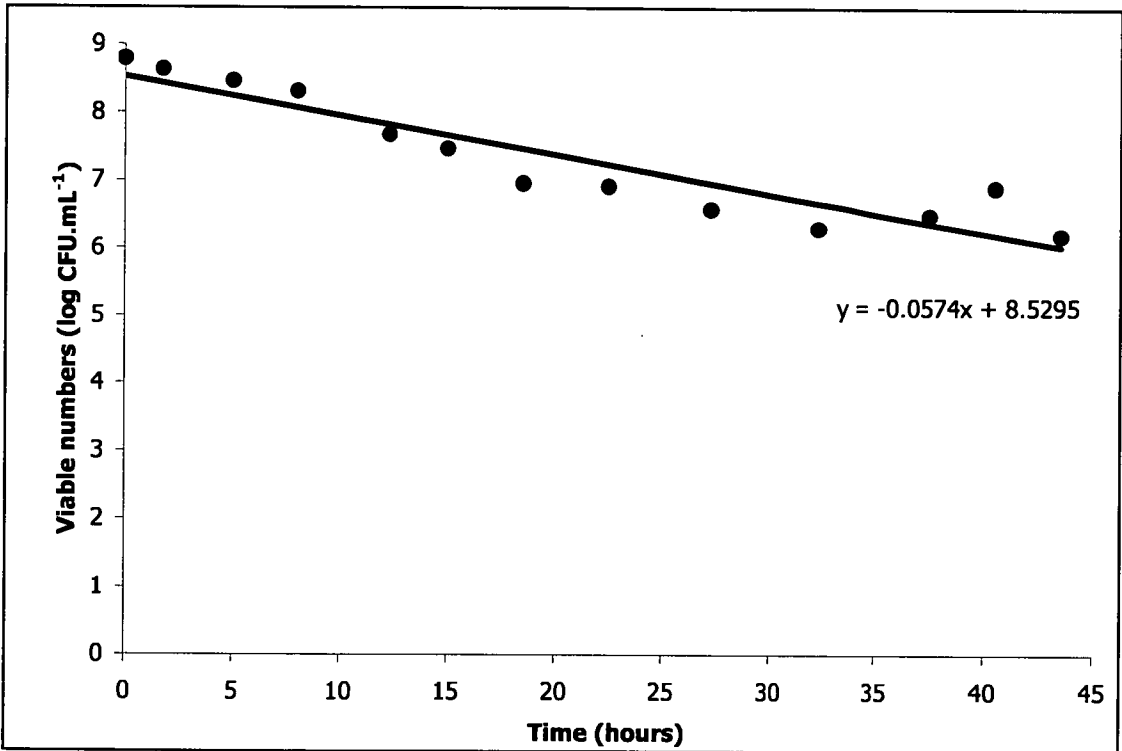
**Figure B.29** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.92 and with the lines of best fit to the data and equations shown, under standard oxygen conditions (●, —;  $a_w$  0.926, pH 6.05, without lactic acid; or ×, ---;  $a_w$  0.927, pH 6.05, without lactic acid). Viable cells were enumerated on BHA-P at 37°C for 14 hours.



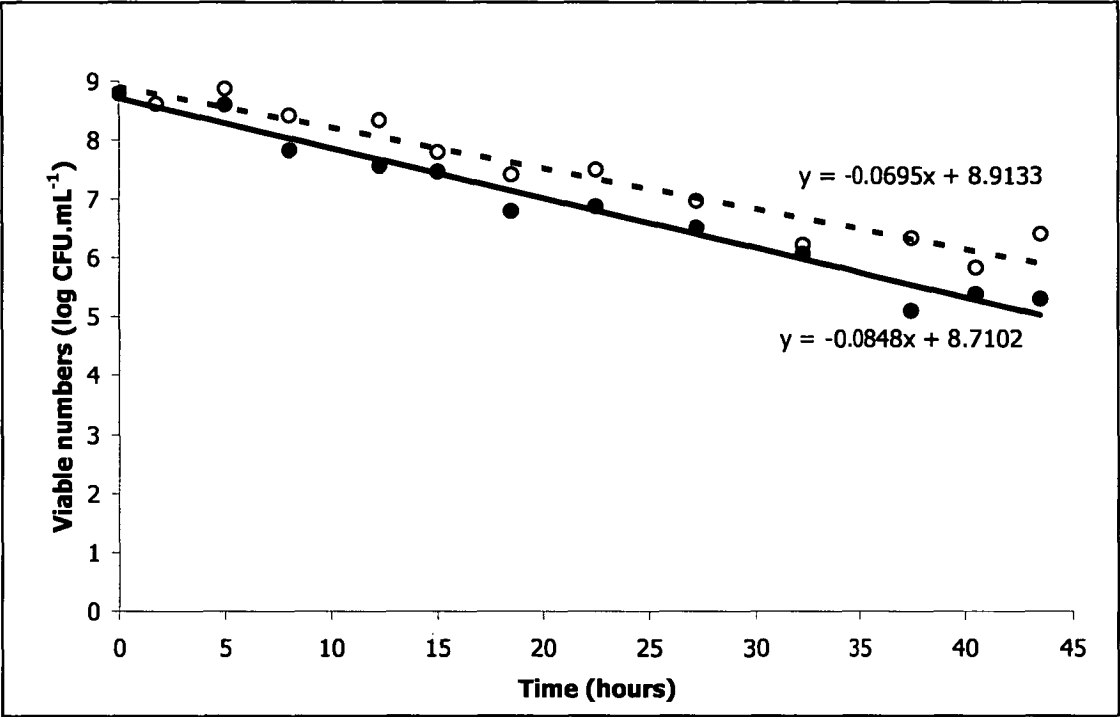
**Figure B.30** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.91 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.911, pH 6.00, without lactic acid) or reduced (○, ---;  $a_w$  0.913, pH 6.05, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



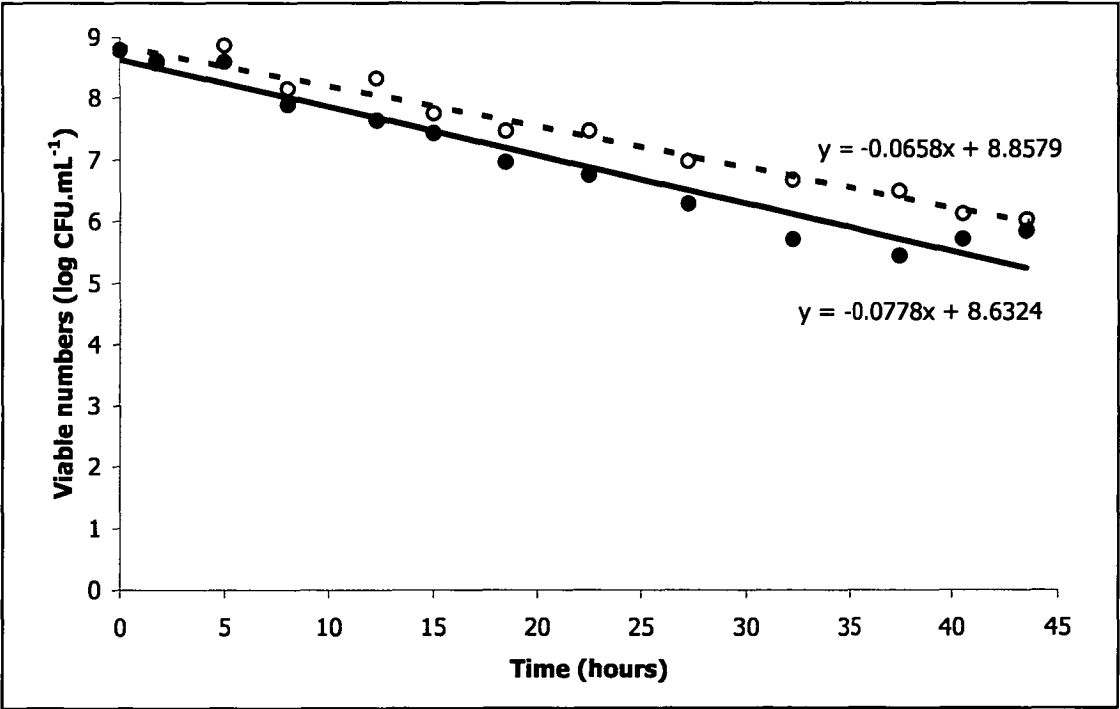
**Figure B.31** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.91 under reduced oxygen conditions ( $a_w$  0.911, pH 6.00, without lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



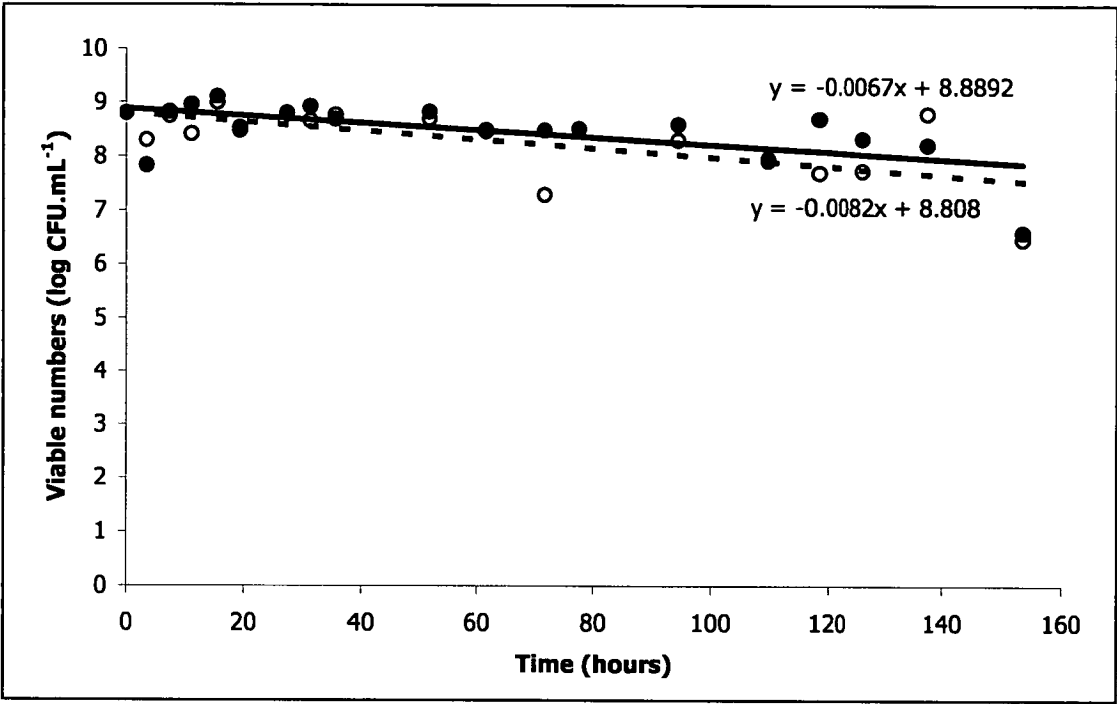
**Figure B.32** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with  $a_w$  0.90 under standard oxygen conditions ( $a_w$  0.909, pH 6.05, without lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



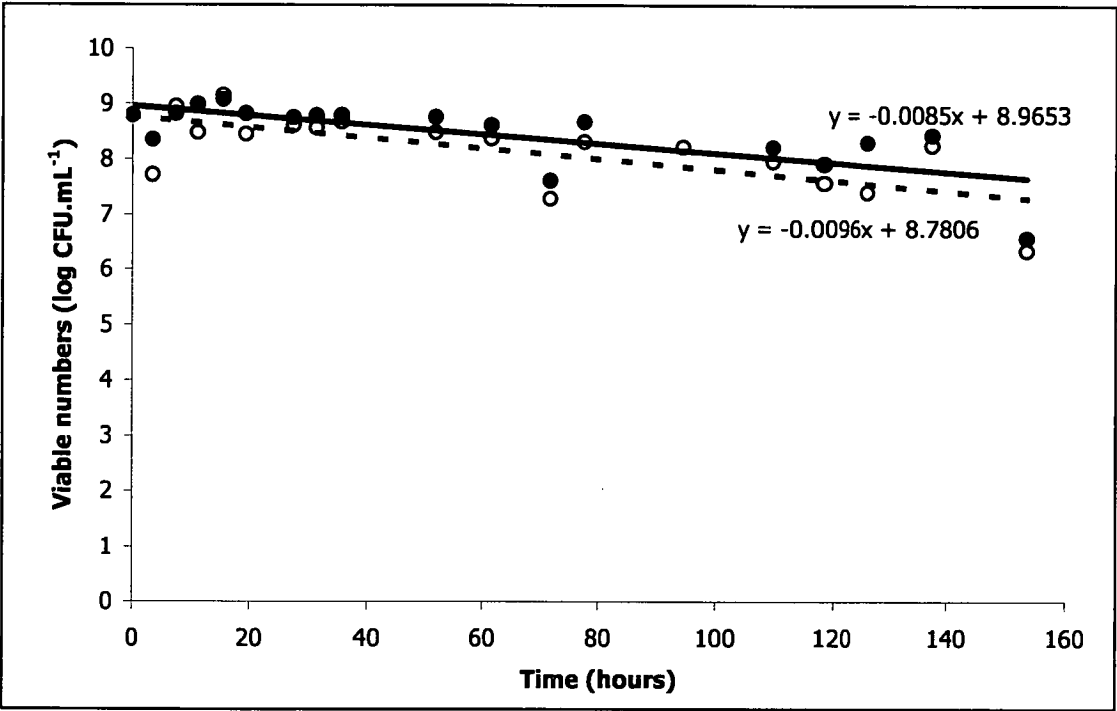
**Figure B.33** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with a<sub>w</sub> 0.89 and with lines of best fit to the data and equations shown, under standard (●, —; a<sub>w</sub> 0.896, pH 6.03, without lactic acid) or reduced (○, ---; a<sub>w</sub> 0.895, pH 5.78, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



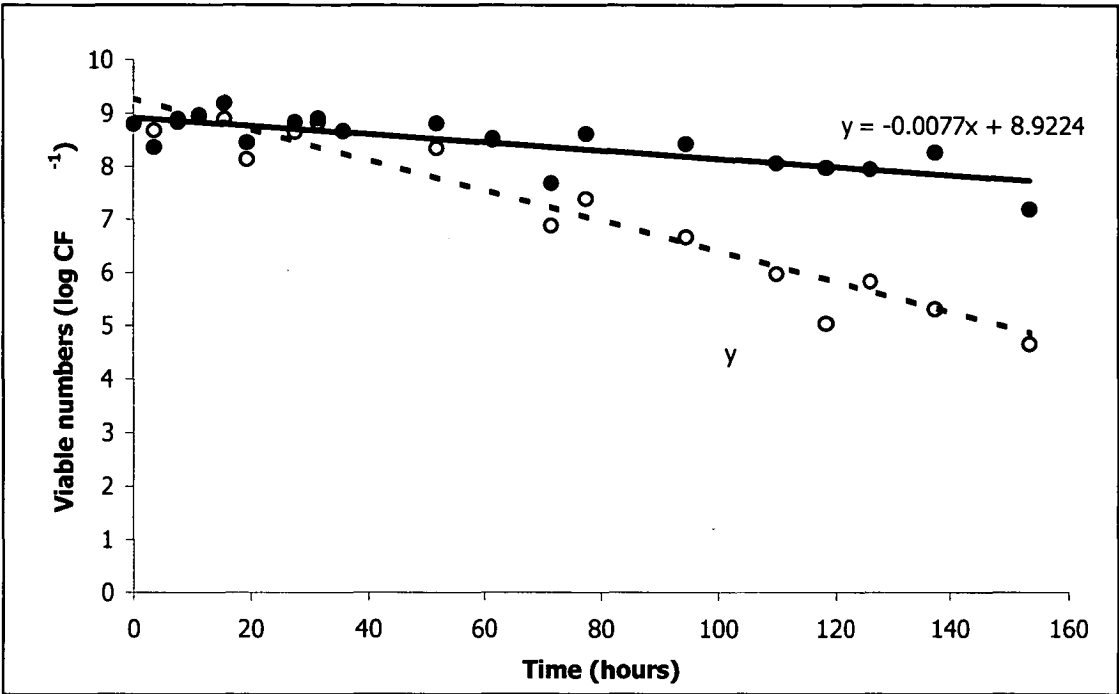
**Figure B.34** Inactivation kinetics of *E. coli* M23 in the broth model at 35°C, with a<sub>w</sub> 0.89 and with the lines of best fit to the data and equations shown, under standard (●, —; a<sub>w</sub> 0.891, pH 5.97, without lactic acid) or reduced (○, ---; a<sub>w</sub> 0.892, pH 5.76, without lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



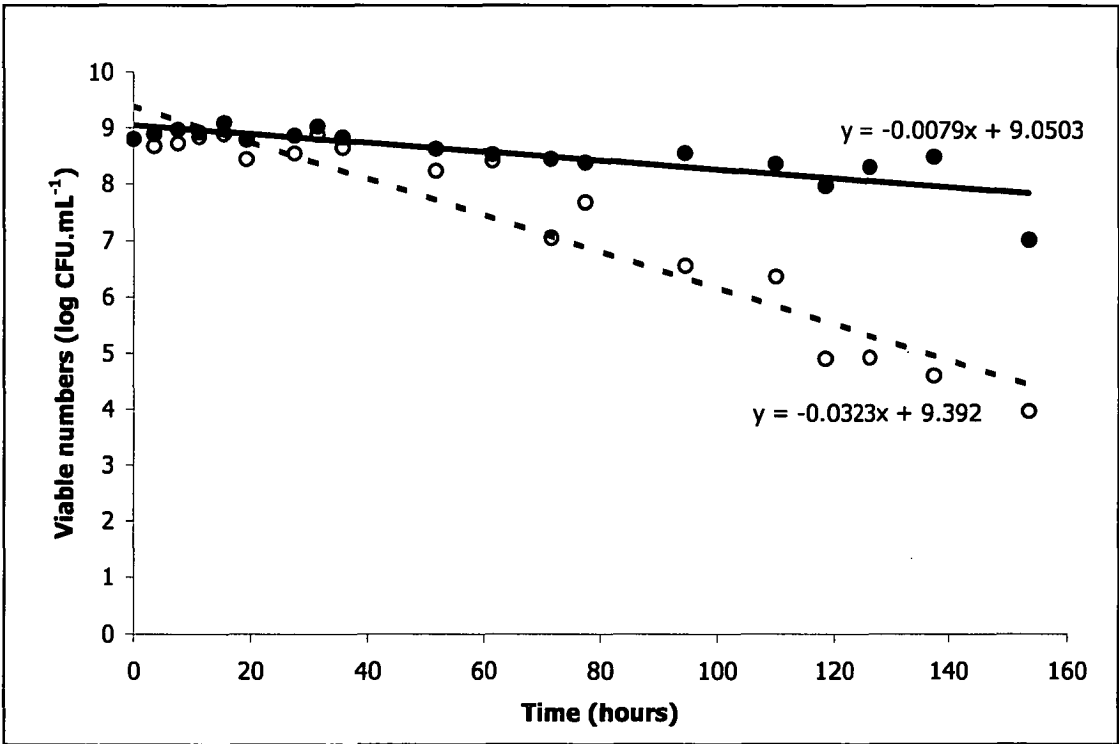
**Figure B.35** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.95 and with lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.954, pH 5.08, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.954, pH 4.98, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.3.



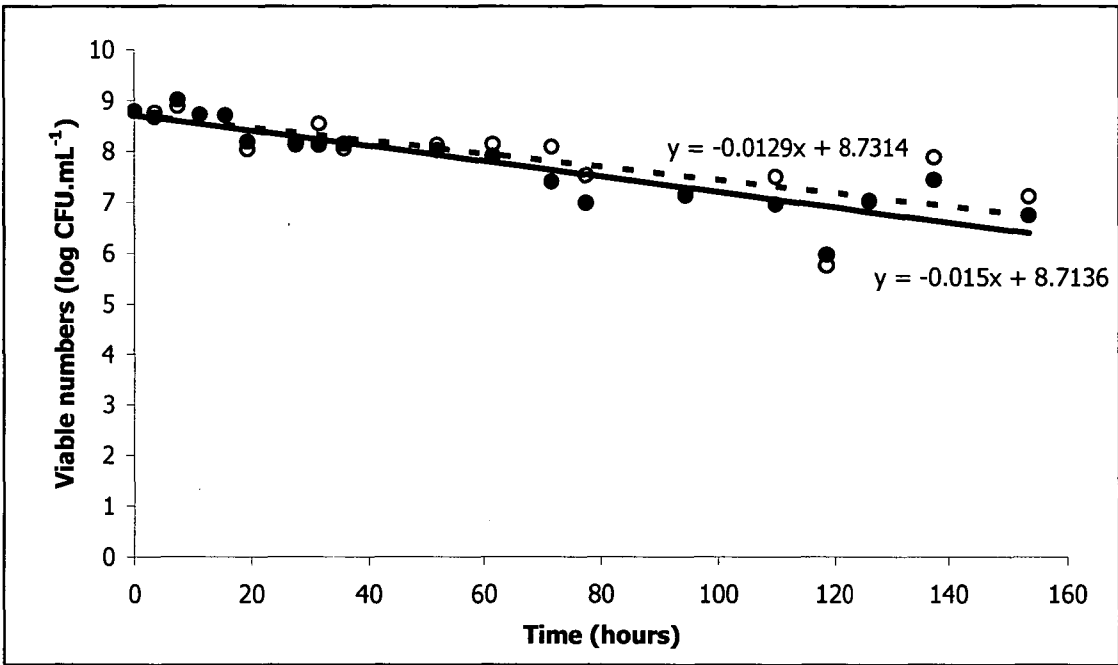
**Figure B.36** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.95 and with lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.954, pH 5.08, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.954, pH 4.98, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



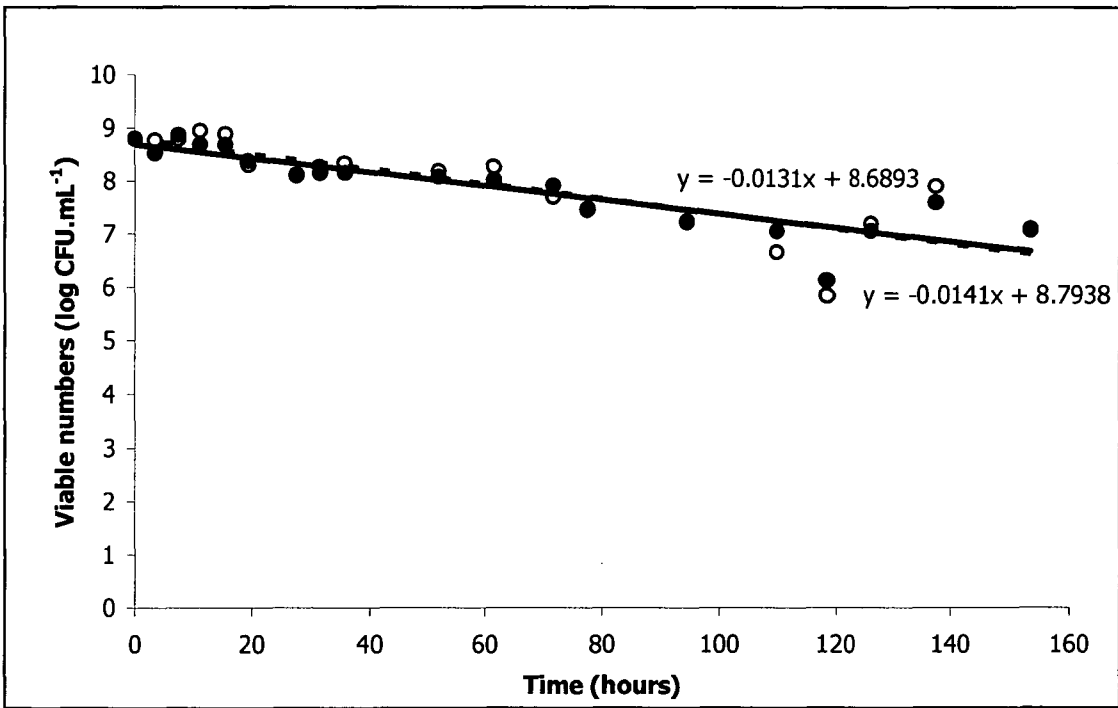
**Figure B.37** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.95 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.954, pH 4.75, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.954, pH 4.73, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



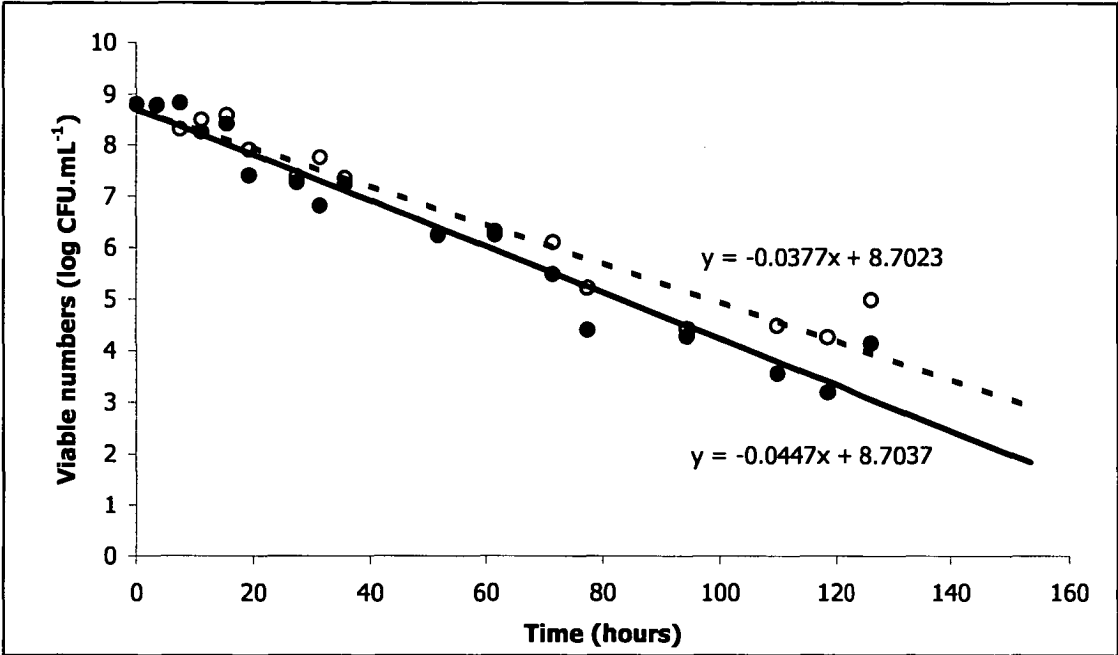
**Figure B.38** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.95 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.954, pH 4.75, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.954, pH 4.73, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



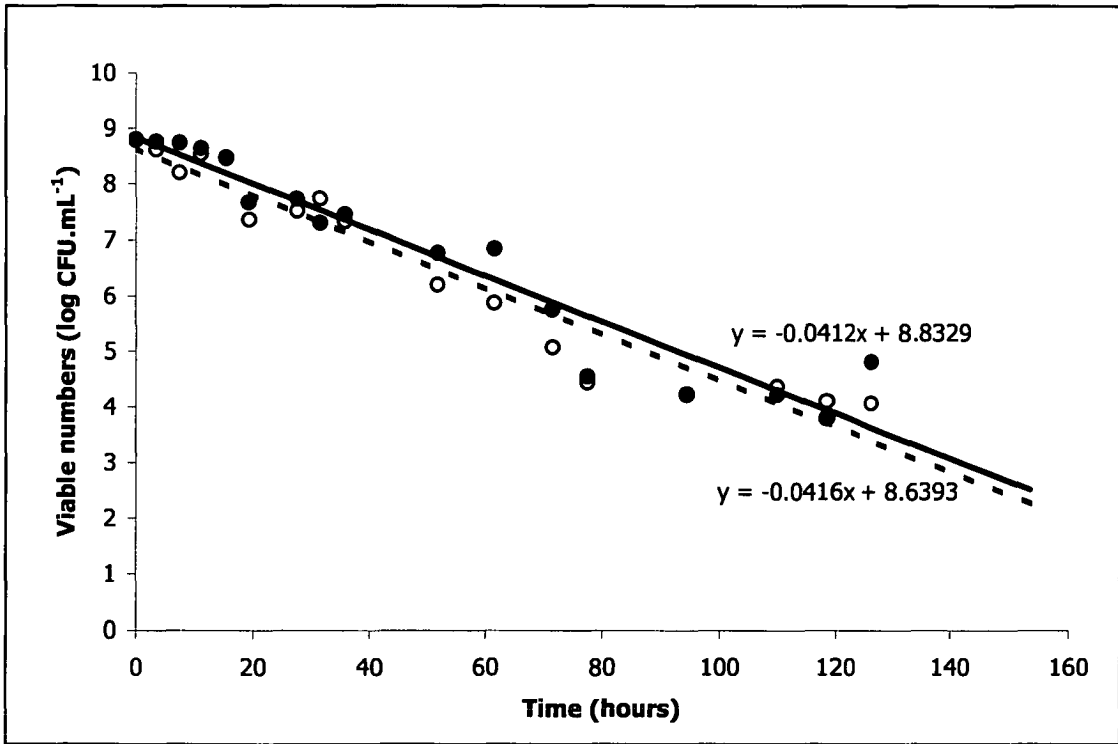
**Figure B.39** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.91 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.911, pH 4.93, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.914, pH 5.02, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



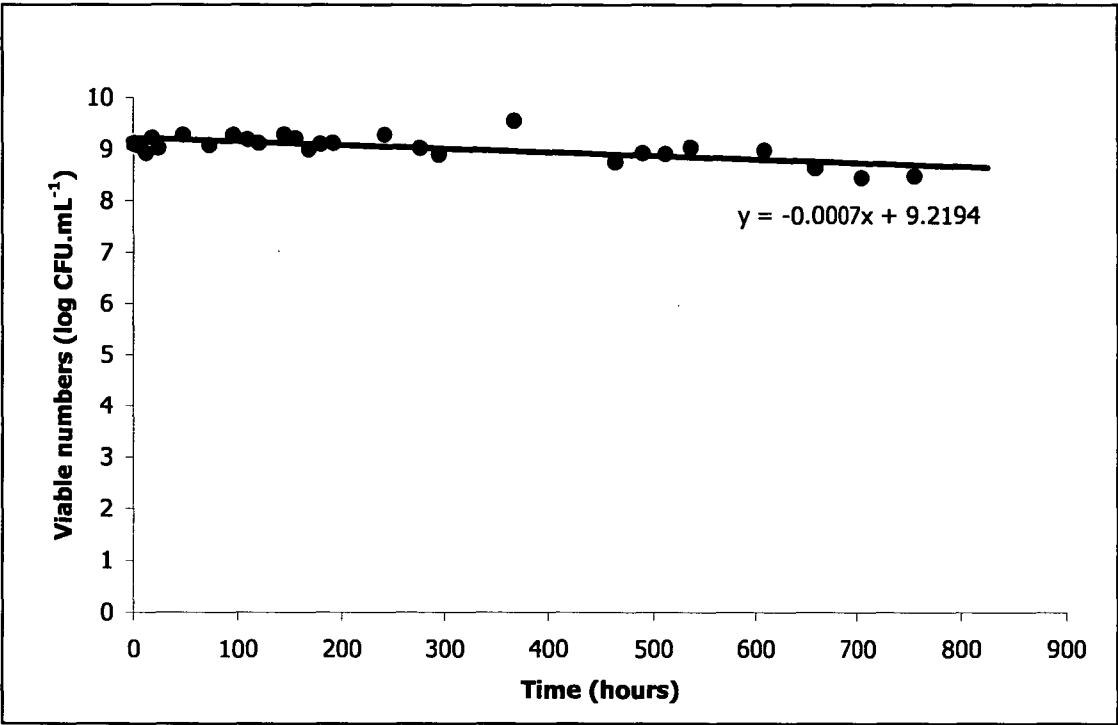
**Figure B.40** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.91 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.913, pH 4.93, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.914, pH 5.02, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



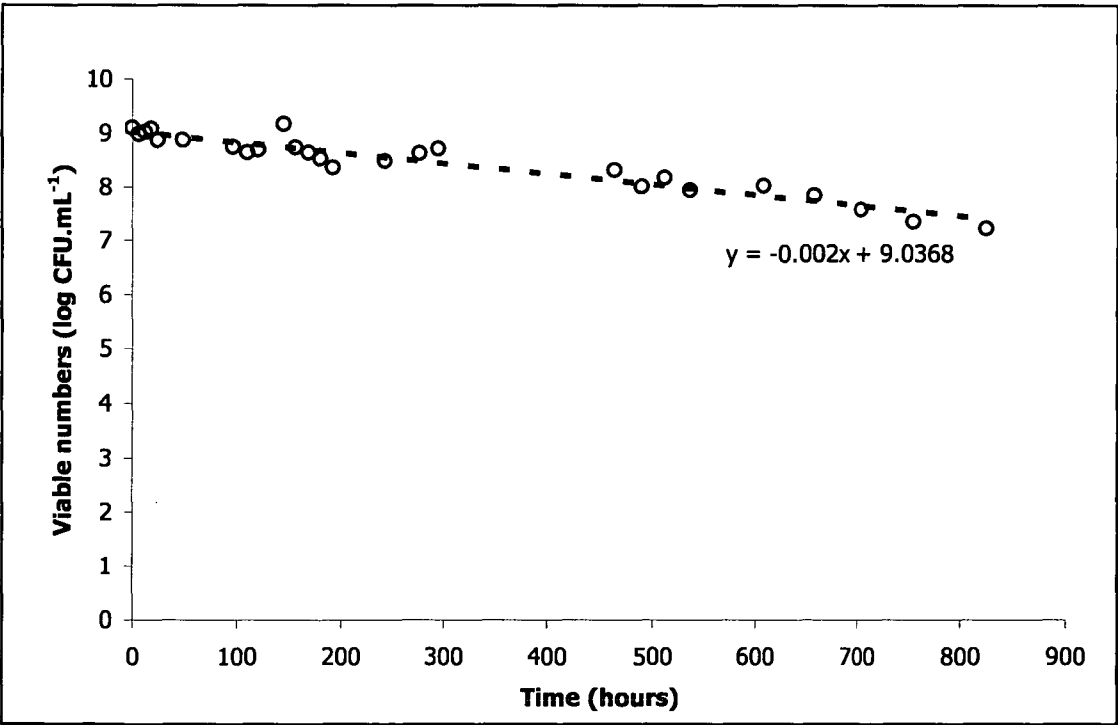
**Figure B.41** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.86 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.864, pH 4.95, lactic acid 150 mM) or reduced (○, ---;  $a_w$  0.868, pH 4.82, lactic acid 150 mM) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure B.42** Inactivation kinetics of *E. coli* M23 in the broth model at 20°C, with  $a_w$  0.86 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.864, pH 4.95, lactic acid 150 mM) or reduced (○, ---;  $a_w$  0.868, pH 4.82, lactic acid 150 mM) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

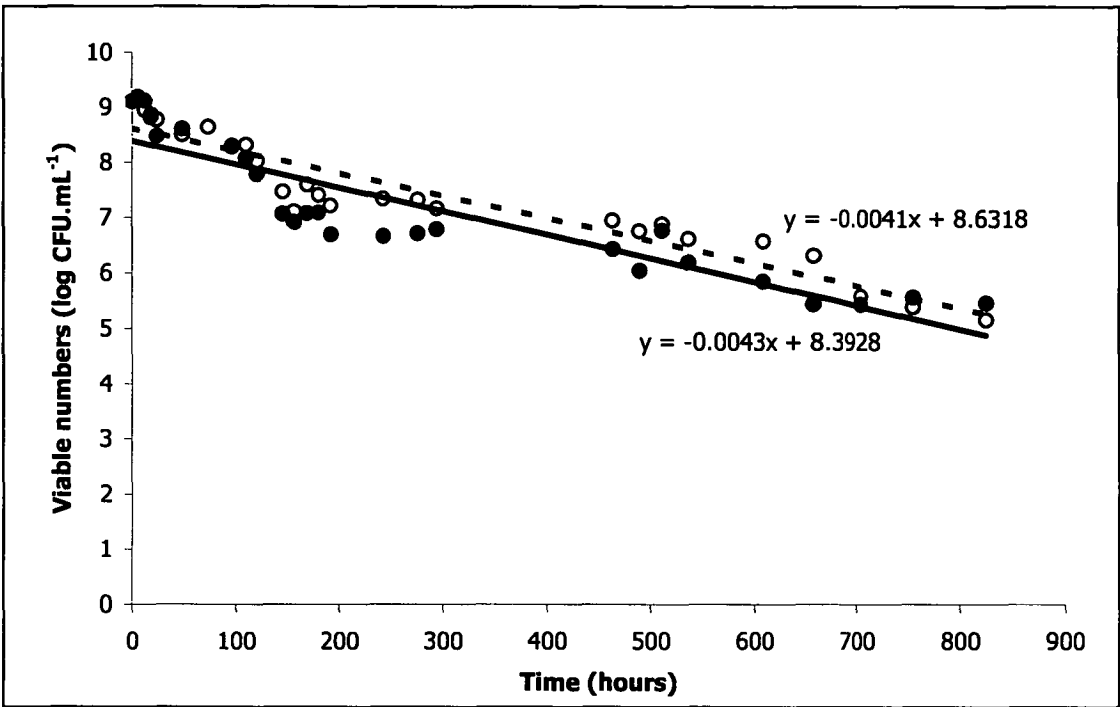


**Figure B.43** Inactivation kinetics of *E. coli* M23 in the broth model at 10°C, with  $a_w$  0.96 under standard oxygen conditions ( $a_w$  0.962, pH 5.20, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

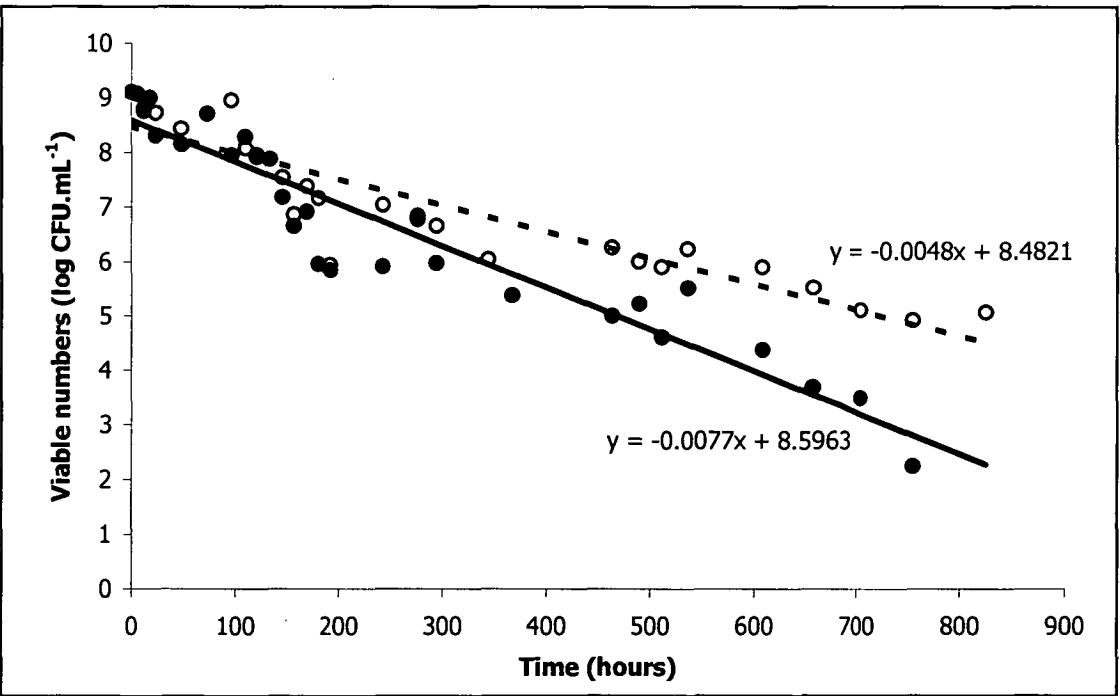


**Figure B.44** Inactivation kinetics of *E. coli* M23 in the broth model at 10°C, with  $a_w$  0.95 under reduced oxygen conditions ( $a_w$  0.959, pH 5.33, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.4.

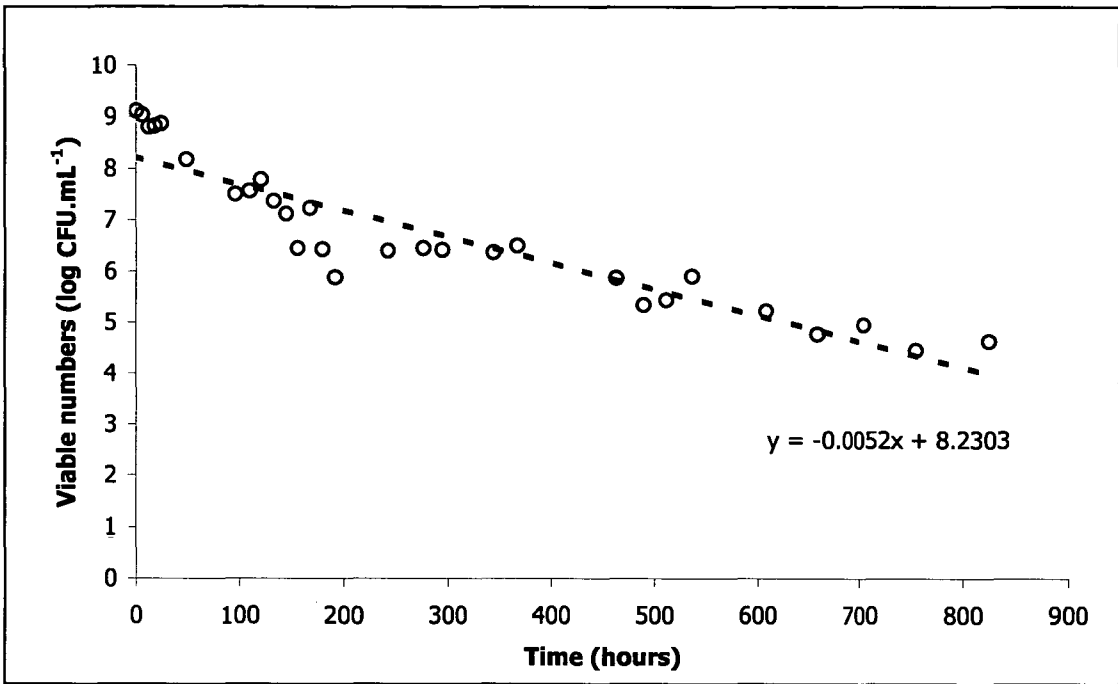




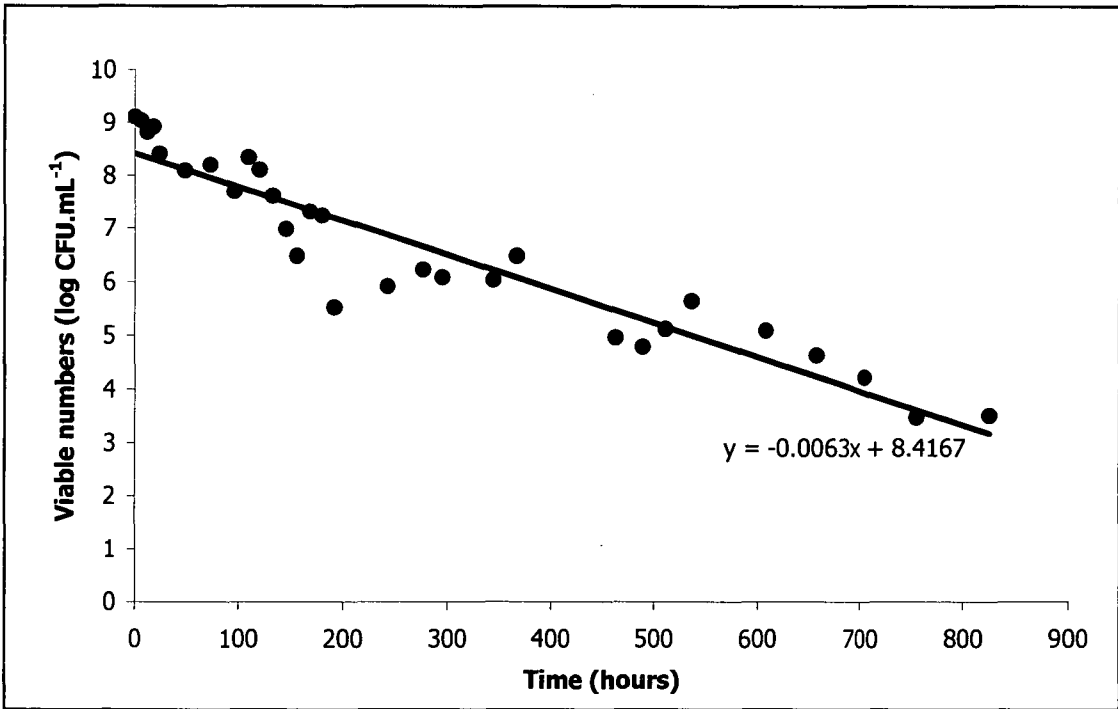
**Figure B.45** Inactivation kinetics of *E. coli* M23 in the broth model at 10°C, with  $a_w$  0.92 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.922, pH 5.08, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.926, pH 5.26, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



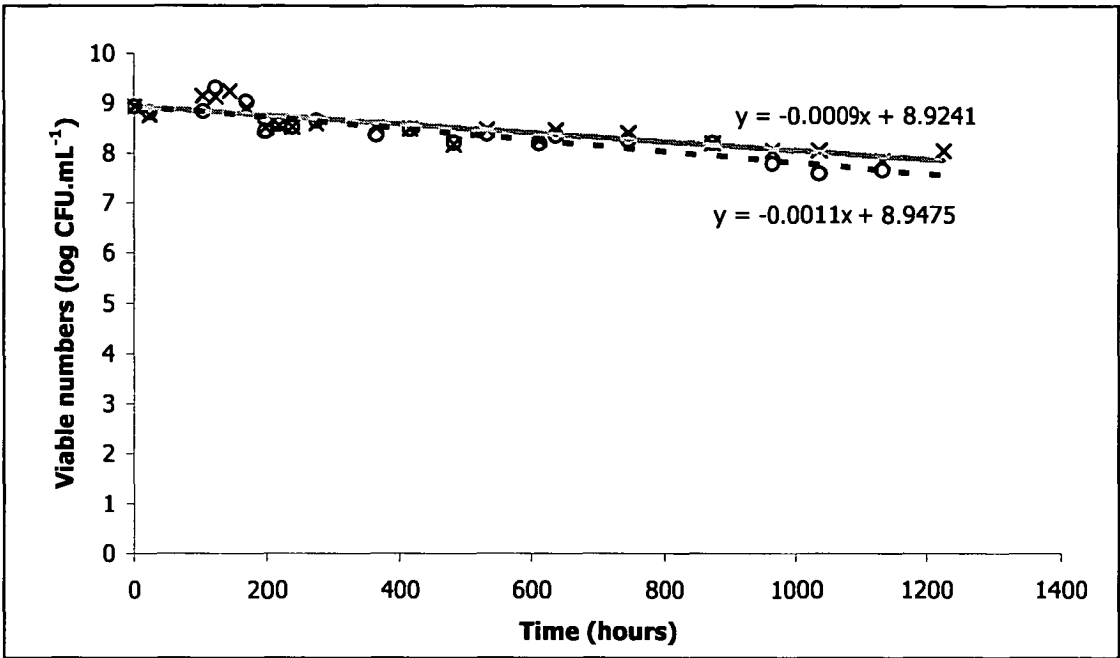
**Figure B.46** Inactivation kinetics of *E. coli* M23 in the broth model at 10°C, with  $a_w$  0.89 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.893, pH 5.10, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.894, pH 5.22, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



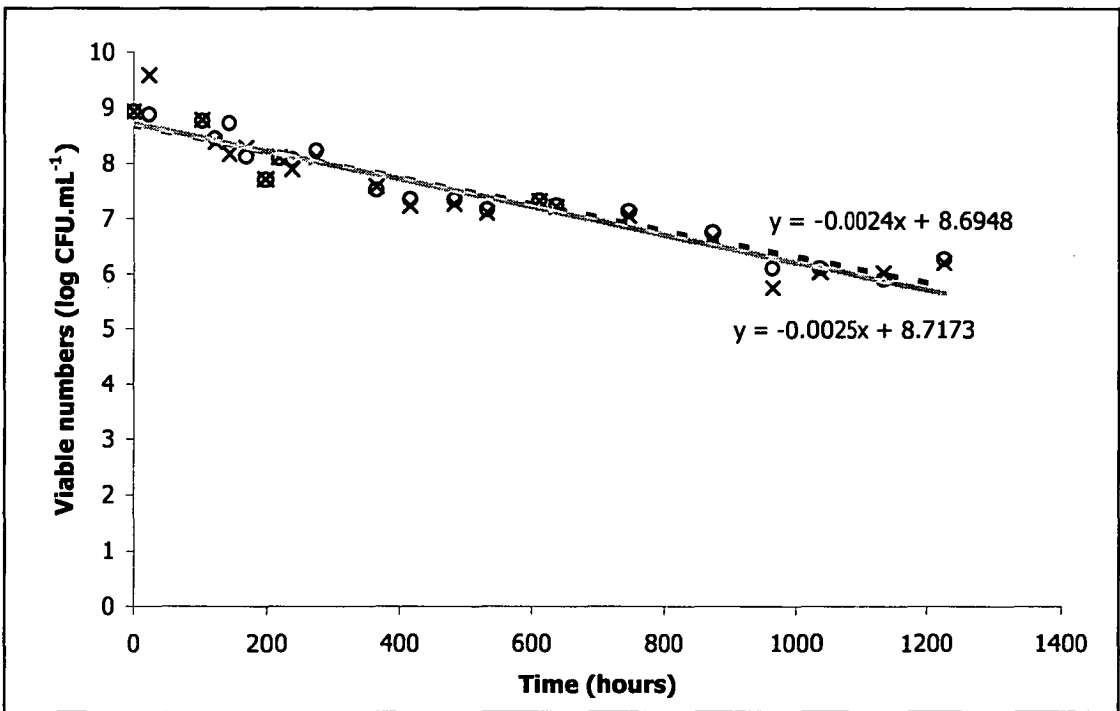
**Figure B.47** Inactivation kinetics of *E. coli* M23 in the broth model at 10°C, with  $a_w$  0.88 under reduced oxygen conditions ( $a_w$  0.888, pH 5.33, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



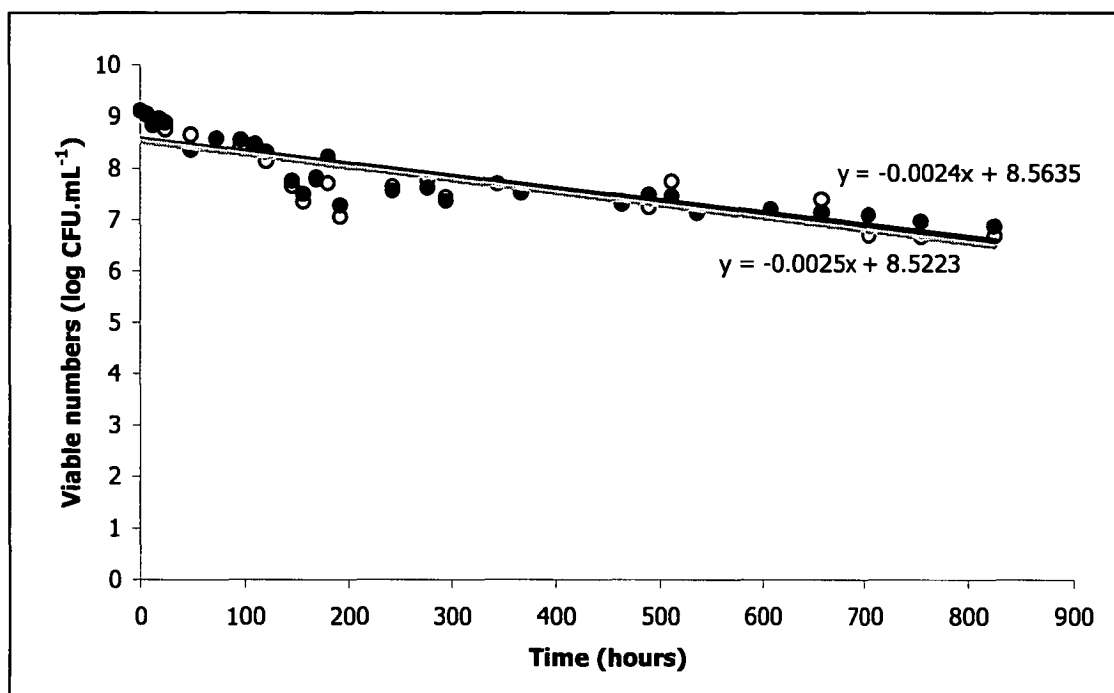
**Figure B.48** Inactivation kinetics of *E. coli* M23 in the broth model at 10°C, with  $a_w$  0.87 under standard oxygen conditions ( $a_w$  0.879, pH 5.11, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.4.



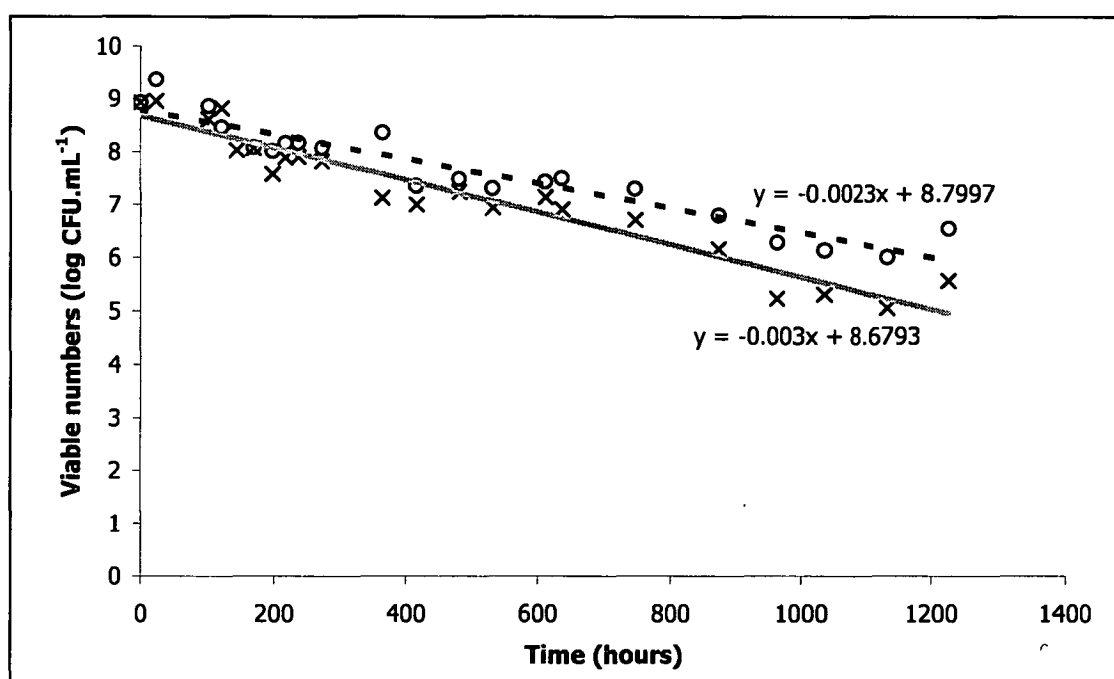
**Figure B.49** Inactivation kinetics of *E. coli* M23 in the broth model at 5°C, with  $a_w$  0.96, under reduced oxygen conditions and with the lines of best fit to the data and equations shown (O, ---;  $a_w$  0.967, pH 5.03, 150 mM lactic acid; or X, —;  $a_w$  0.964, pH 4.99, 150 mM lactic acid). Viable cells were enumerated on BHA-P at 37°C for 14 hours. Data depicted previously in Figure B.5.



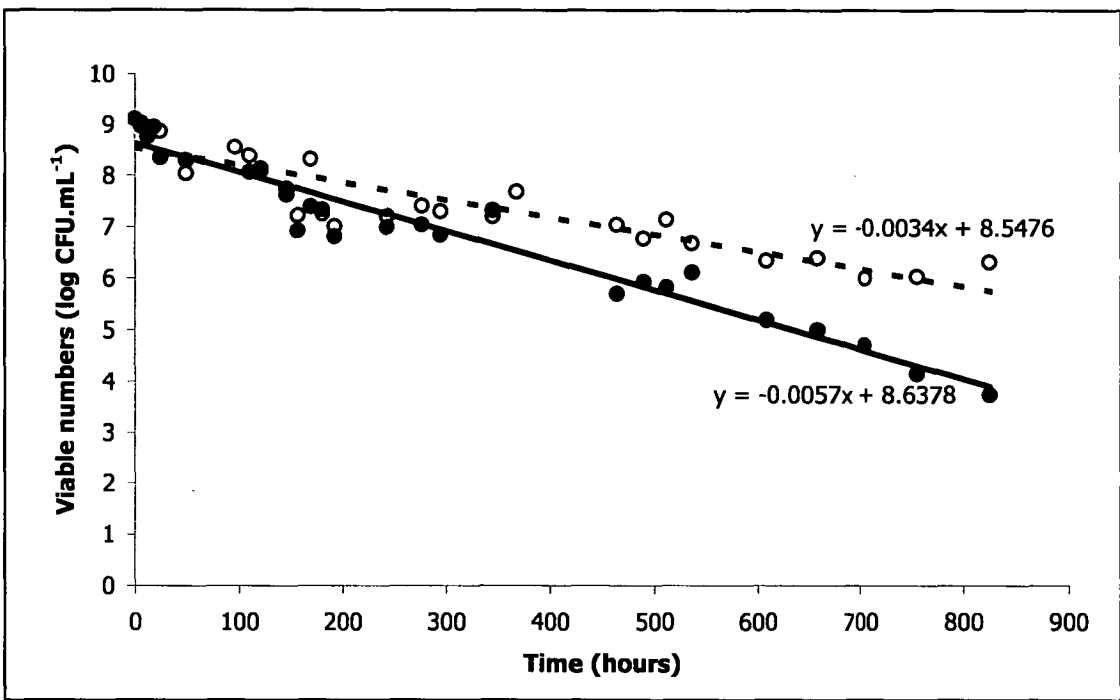
**Figure B.50** Inactivation kinetics of *E. coli* M23 in the broth model at 5°C, with  $a_w$  0.93, under reduced oxygen conditions and with the lines of best fit to the data and equations shown (O, ---;  $a_w$  0.933, pH 5.03, 150 mM lactic acid; or X, —;  $a_w$  0.932, pH 4.95, 150 mM lactic acid). Viable cells were enumerated on BHA-P at 37°C for 14 hours.



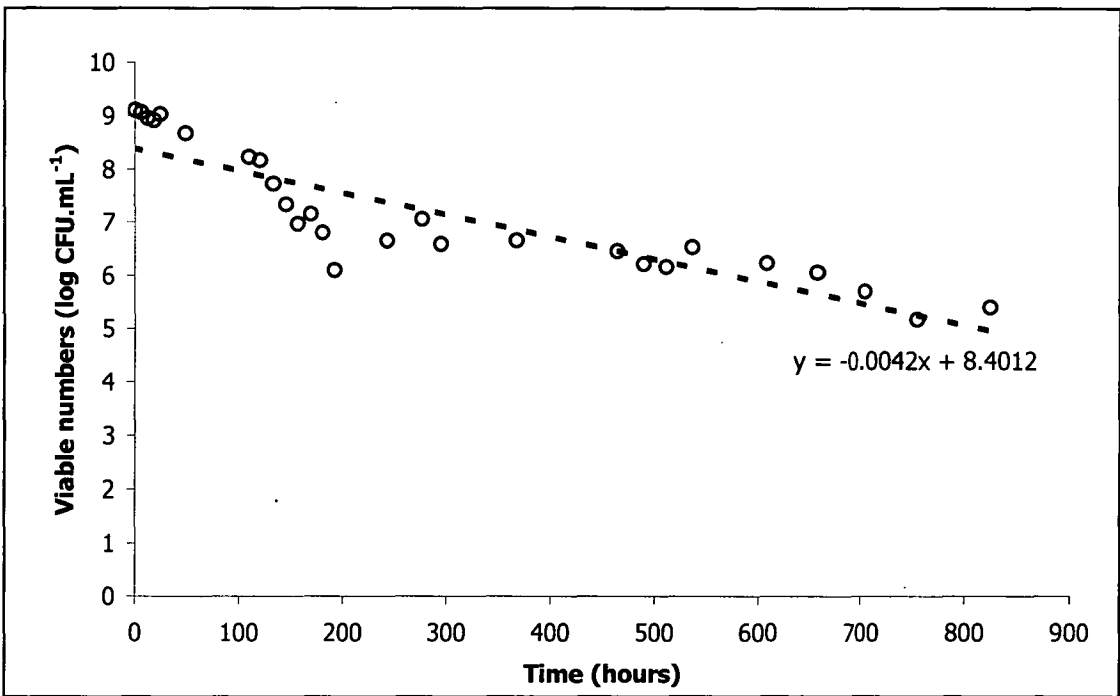
**Figure B.51** Inactivation kinetics of *E. coli* M23 in the broth model at 5°C, with  $a_w$  0.92 and with the lines of best fit to the data and equations shown, under standard (●, —;  $a_w$  0.921, pH 5.17, 150 mM lactic acid) or reduced (○, ---;  $a_w$  0.922, pH 5.33, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



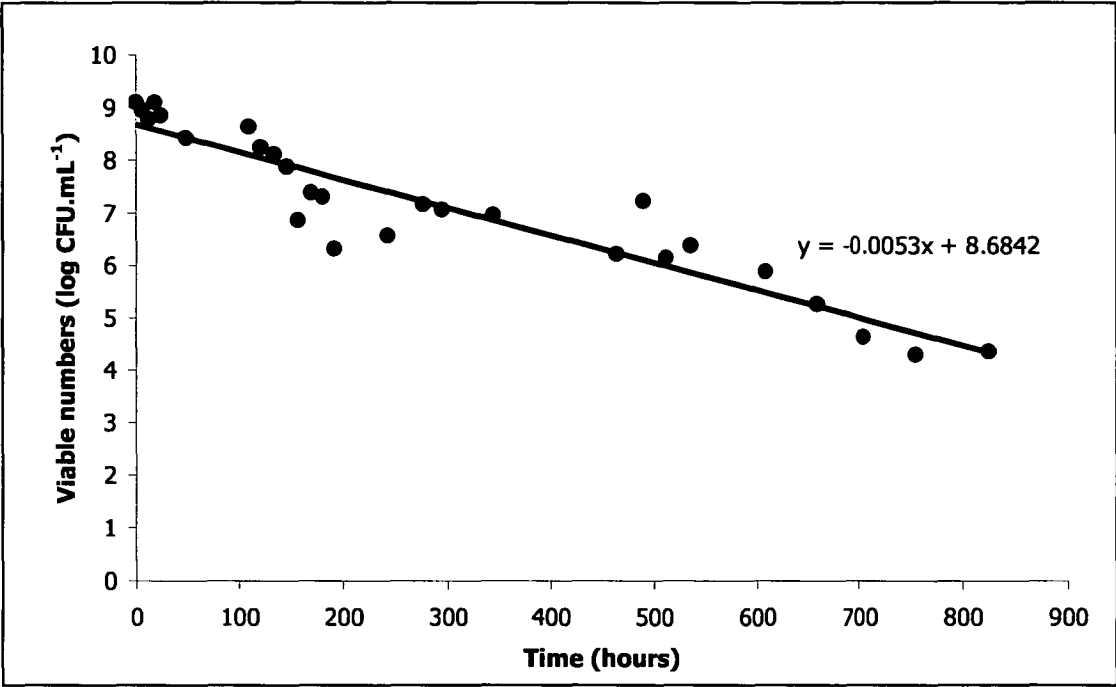
**Figure B.52** Inactivation kinetics of *E. coli* M23 in the broth model at 5°C, with  $a_w$  0.92, under reduced oxygen conditions and with the lines of best fit to the data and equations shown (○, ---;  $a_w$  0.924, pH 5.00, 150 mM lactic acid; or ×, —;  $a_w$  0.922, pH 5.05, 150 mM lactic acid). Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure B.53** Inactivation kinetics of *E. coli* M23 in the broth model at 5°C, with a<sub>w</sub> 0.89, with the lines of best fit to the data and equations shown, under standard (●; a<sub>w</sub> 0.893, pH 5.13, 150 mM lactic acid) or reduced (○; a<sub>w</sub> 0.893, pH 5.23, 150 mM lactic acid) oxygen conditions. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure B.54** Inactivation kinetics of *E. coli* M23 in the broth model at 5°C, with a<sub>w</sub> 0.88 under reduced oxygen conditions (a<sub>w</sub> 0.881, pH 5.27, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.



**Figure B.55** Inactivation kinetics of *E. coli* M23 in the broth model at 5°C, with  $a_w$  0.87 under standard oxygen conditions ( $a_w$  0.870, pH 5.10, 150 mM lactic acid). The line of best fit to the data and equation is shown. Viable cells were enumerated on BHA-P at 37°C for 14 hours.

**B.2.2 Combination of Conditions Tested and the Observed Rates of Inactivation of *Escherichia coli***

The inactivation rates of *E. coli* in response to combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability (standard or reduced) in the broth model was calculated from the lines of best fit to the data, as shown in Appendix B.2.1. This set of 62 inactivation rate data is tabulated below (Table B.2).

**Table B.2** The combinations of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability tested in the broth model and the observed inactivation rates of *E. coli* calculated from the line of best fit to the data shown in Appendix B.2.1.

| Temp.<br>(°C) | $a_w$ | pH   | Lactic acid<br>concentration<br>(mM) | Oxygen<br>availability | Inactivation<br>rate <sup>a</sup> (log<br>CFU.mL <sup>-1</sup> .hr <sup>-1</sup> ) | see<br>Figure | Curve<br>type <sup>b</sup> |
|---------------|-------|------|--------------------------------------|------------------------|--|---------------|----------------------------|
| 55            | 0.971 | 4.14 | 150                                  | Standard               | 7.88   | B16           | III                        |
| 55            | 0.962 | 4.55 | 150                                  | Standard               | 7.11   | B17           | II                         |
| 55            | 0.929 | 4.90 | 150                                  | Standard               | 13.37  | B18           | II                         |
| 55            | 0.873 | 4.71 | 150                                  | Standard               | 14.05  | B19           | III                        |
| 45            | 0.950 | 5.66 | 150                                  | Reduced                | 0.11   | B20           | III                        |
| 45            | 0.947 | 5.22 | 150                                  | Standard               | 0.12   | B21           | III                        |
| 45            | 0.928 | 4.97 | 150                                  | Reduced                | 0.68   | B22           | II                         |
| 45            | 0.895 | 5.10 | 150                                  | Reduced                | 0.67   | B23           | II                         |
| 35            | 0.950 | 5.74 | 150                                  | Reduced                | 0.025  | B24           | III                        |
| 35            | 0.945 | 5.25 | 150                                  | Standard               | 0.021  | B25           | III                        |
| 35            | 0.944 | 5.94 | 0                                    | Reduced                | 0.038  | B26           | I <sup>c</sup>             |
| 35            | 0.943 | 5.84 | 0                                    | Reduced                | 0.039  | B27           | I <sup>c</sup>             |
| 35            | 0.941 | 6.15 | 0                                    | Standard               | 0.026  | B26           | I <sup>c</sup>             |
| 35            | 0.940 | 6.13 | 0                                    | Standard               | 0.031  | B27           | I <sup>c</sup>             |
| 35            | 0.932 | 5.74 | 0                                    | Reduced                | 0.050  | B28           | I <sup>c</sup>             |
| 35            | 0.930 | 5.57 | 0                                    | Reduced                | 0.049  | B28           | I <sup>c</sup>             |
| 35            | 0.927 | 6.05 | 0                                    | Standard               | 0.040  | B29           | I <sup>c</sup>             |
| 35            | 0.926 | 6.05 | 0                                    | Standard               | 0.036  | B29           | III                        |
| 35            | 0.913 | 6.05 | 0                                    | Reduced                | 0.058  | B30           | I                          |
| 35            | 0.911 | 6.00 | 0                                    | Standard               | 0.065  | B30           | III                        |
| 35            | 0.911 | 6.00 | 0                                    | Reduced                | 0.069  | B31           | I                          |
| 35            | 0.909 | 6.05 | 0                                    | Standard               | 0.057  | B32           | III                        |
| 35            | 0.896 | 6.03 | 0                                    | Standard               | 0.085  | B33           | III                        |
| 35            | 0.895 | 5.78 | 0                                    | Reduced                | 0.070  | B33           | I                          |

(Table B.2 continued ...)

| Temp.<br>(°C) | a <sub>w</sub> | pH   | Lactic acid<br>concentration<br>(mM) | Oxygen<br>availability | Inactivation<br>rate <sup>a</sup> (log<br>CFU.mL <sup>-1</sup> .hr <sup>-1</sup> ) | see<br>Figure | Curve<br>type <sup>b</sup> |
|---------------|----------------|------|--------------------------------------|------------------------|--|---------------|----------------------------|
| 35            | 0.892          | 5.76 | 0                                    | Reduced                | 0.066  | B34           | III                        |
| 35            | 0.891          | 5.97 | 0                                    | Standard               | 0.078  | B34           | III                        |
| 20            | 0.954          | 5.08 | 150                                  | Standard               | 0.0085   | B36           | I <sup>c</sup>             |
| 20            | 0.954          | 5.08 | 150                                  | Standard               | 0.0067   | B35           | I <sup>c</sup>             |
| 20            | 0.954          | 4.98 | 150                                  | Reduced                | 0.0096   | B36           | III                        |
| 20            | 0.954          | 4.98 | 150                                  | Reduced                | 0.0082   | B35           | I <sup>c</sup>             |
| 20            | 0.954          | 4.75 | 150                                  | Standard               | 0.0079   | B38           | I <sup>c</sup>             |
| 20            | 0.954          | 4.75 | 150                                  | Standard               | 0.0077   | B37           | I <sup>c</sup>             |
| 20            | 0.954          | 4.73 | 150                                  | Reduced                | 0.032  | B38           | II                         |
| 20            | 0.954          | 4.73 | 150                                  | Reduced                | 0.029  | B37           | II                         |
| 20            | 0.914          | 5.02 | 150                                  | Reduced                | 0.014  | B40           | III                        |
| 20            | 0.914          | 5.02 | 150                                  | Reduced                | 0.013  | B39           | III                        |
| 20            | 0.913          | 4.93 | 150                                  | Standard               | 0.013  | B40           | III                        |
| 20            | 0.911          | 4.93 | 150                                  | Standard               | 0.015  | B39           | III                        |
| 20            | 0.868          | 4.82 | 150                                  | Reduced                | 0.042  | B42           | III                        |
| 20            | 0.868          | 4.82 | 150                                  | Reduced                | 0.038  | B41           | III                        |
| 20            | 0.864          | 4.95 | 150                                  | Standard               | 0.045  | B42           | III                        |
| 20            | 0.864          | 4.95 | 150                                  | Standard               | 0.041  | B41           | I                          |
| 10            | 0.962          | 5.20 | 150                                  | Standard               | 0.00070  | B43           | II                         |
| 10            | 0.959          | 5.33 | 150                                  | Reduced                | 0.0020   | B44           | I <sup>c</sup>             |
| 10            | 0.926          | 5.26 | 150                                  | Reduced                | 0.0041   | B45           | III                        |
| 10            | 0.922          | 5.08 | 150                                  | Standard               | 0.0043   | B45           | III                        |
| 10            | 0.894          | 5.22 | 150                                  | Reduced                | 0.0048   | B46           | III                        |
| 10            | 0.893          | 5.10 | 150                                  | Standard               | 0.0077   | B46           | III                        |
| 10            | 0.888          | 5.33 | 150                                  | Reduced                | 0.0052   | B47           | III                        |



(Table B.2 continued ...)

| Temp.<br>(°C) | $a_w$ | pH   | Lactic acid<br>concentration<br>(mM) | Oxygen<br>availability | Inactivation<br>rate <sup>a</sup> (log<br>CFU.mL <sup>-1</sup> .hr <sup>-1</sup> ) | see<br>Figure | Curve<br>type <sup>b</sup> |
|---------------|-------|------|--------------------------------------|------------------------|--|---------------|----------------------------|
| 10            | 0.879 | 5.11 | 150                                  | Standard               | 0.0063   | B48           | III                        |
| 5             | 0.967 | 5.03 | 150                                  | Reduced                | 0.0011   | B49           | I <sup>c</sup>             |
| 5             | 0.964 | 4.99 | 150                                  | Reduced                | 0.00090  | B49           | I <sup>c</sup>             |
| 5             | 0.933 | 5.03 | 150                                  | Reduced                | 0.0024   | B50           | III                        |
| 5             | 0.932 | 4.95 | 150                                  | Reduced                | 0.0025   | B50           | III                        |
| 5             | 0.924 | 5.00 | 150                                  | Reduced                | 0.0023   | B52           | III                        |
| 5             | 0.922 | 5.33 | 150                                  | Reduced                | 0.0025   | B51           | III                        |
| 5             | 0.922 | 5.05 | 150                                  | Reduced                | 0.0030   | B52           | III                        |
| 5             | 0.921 | 5.17 | 150                                  | Standard               | 0.0024   | B51           | III                        |
| 5             | 0.893 | 5.23 | 150                                  | Reduced                | 0.0034   | B53           | III                        |
| 5             | 0.893 | 5.13 | 150                                  | Standard               | 0.0057   | B53           | III                        |
| 5             | 0.881 | 5.27 | 150                                  | Standard               | 0.0042   | B54           | III                        |
| 5             | 0.870 | 5.10 | 150                                  | Standard               | 0.0053   | B55           | III                        |

<sup>a</sup> Overall inactivation rate based on the line of best fit to the data assuming log-linear inactivation.<sup>b</sup> see Appendix B.3.<sup>c</sup> Inactivation kinetics were monitored for  $\leq 2$ -log kill in these Type I survival curves.

### B.3 DESCRIBING INACTIVATION RATES USING THE BROTH MODEL

To simplify the summary of the inactivation curve data given in Appendices B.1.1 and B.2.1, inactivation rates overall were taken to be log-linear. Close inspection of the inactivation kinetics reveals that some survival curves exhibited biphasic decline (i.e. two phases of log-linear inactivation) and, thus, these could be characterised by two rates of inactivation. The y-intercepts of the log-linear equation fitted to the data were indicative of the type of survival curve (i.e. log-linear or multiphasic). At

a starting value of  $9 \log \text{CFU.mL}^{-1}$ , an intercept of  $9 (\pm 0.20) \log \text{CFU.mL}^{-1}$  ('Type I') suggested that the survival curve was well characterised by log-linear decline (for an example see Figure B.27, also depicted in Figure 3.1A). When the intercept was  $> 9.20 \log \text{CFU.mL}^{-1}$  ('Type II'), the survival curve tended to consist of an initial, slow rate of inactivation followed by a more rapid rate of decline (for an example see Figure B.22, also depicted in Figure 3.1D), whereas an intercept of  $< 8.80 \log \text{CFU.mL}^{-1}$  ('Type III') occurred in survival curves characterised by an initial, rapid phase of inactivation that was followed by tailing (for an example see Figure B.54, also depicted in Figure 3.1C).

Of the 62 survival curves given in Appendix B.2.1 (i.e. those used to determine the effect of variables on the inactivation rates of *E. coli* in simulated-UCFM environments; Chapter 4), 19 were Type I, only seven were Type II and the majority (36) were Type III. In all except four cases, Type I survival curves occurred when the inactivation of the population was monitored for  $\leq 2$ -log kill and it is, therefore, likely that had the inactivation of the population been followed for longer a second phase of inactivation would have been observed. Importantly, no correlation between the temperature of incubation,  $a_w$ , pH, lactic acid concentration and/or oxygen availability in the broth model and the existence of Type I, II or III survival curves could be made. Further, the use of multiple inactivation rates did not improve analysis of the effect of temperature,  $a_w$ , pH, lactic acid concentration and oxygen availability (Section 4.2.6) and so the simplified, overall inactivation rate was used in the study of the effect of variables on the inactivation rates of *E. coli* (Chapter 4).

## APPENDIX C: Conditions and Observed Inactivation Rates of *Escherichia coli* in Salami

The inactivation rates of *E. coli* during fermentation in UCFM (salami) with varying temperature, time,  $a_w$  and pH variables were determined from survival curves provided by Dr. Paul Vanderlinde and detailed in Ross *et al.* (2004). This set of 30 inactivation rate data is tabulated below (Table C.1).

**Table C.1** The combinations of temperature, time,  $a_w$  and pH in the UCFM and the observed inactivation rates of *E. coli* calculated from the line of best fit to the data (survival curves) provided by Dr. Paul Vanderlinde and detailed in Ross *et al.* (2004).

| Temperature<br>(°C) | Time<br>(days) | Water<br>activity | pH   | Inactivation rate <sup>a</sup><br>(log CFU.g <sup>-1</sup> .hr <sup>-1</sup> ) |
|---------------------|----------------|-------------------|------|--|
| 30.4                | 4              | 0.964             | 4.57 | 0.0188   |
| 29.3                | 4              | 0.97              | 4.55 | 0.0208   |
| 15.1                | 10             | 0.771             | 5.58 | 0.00545  |
| 14.2                | 30             | 0.943             | 5.13 | 0.00245  |
| 40                  | 3              | 0.8               | 4.9  | 0.0572   |
| 23.6                | 5              | 0.955             | 4.96 | 0.00249  |
| 24.8                | 3.2            | 0.906             | 4.9  | 0.0193   |
| 23.9                | 3.2            | 0.96              | 4.76 | 0.0137   |
| 23.8                | 3.2            | 0.96              | 4.8  | 0.0108   |
| 23.97               | 2              | 0.951             | 5.24 | 0.0168   |
| 23.97               | 2              | 0.963             | 4.98 | 0.0131   |
| 41.5                | 2              | 0.876             | 6.04 | 0.0613   |
| 41                  | 2              | 0.941             | 4.93 | 0.0534   |
| 29                  | 3              | 0.94              | 4.6  | 0.0246   |

(Table C.1 continued ...)

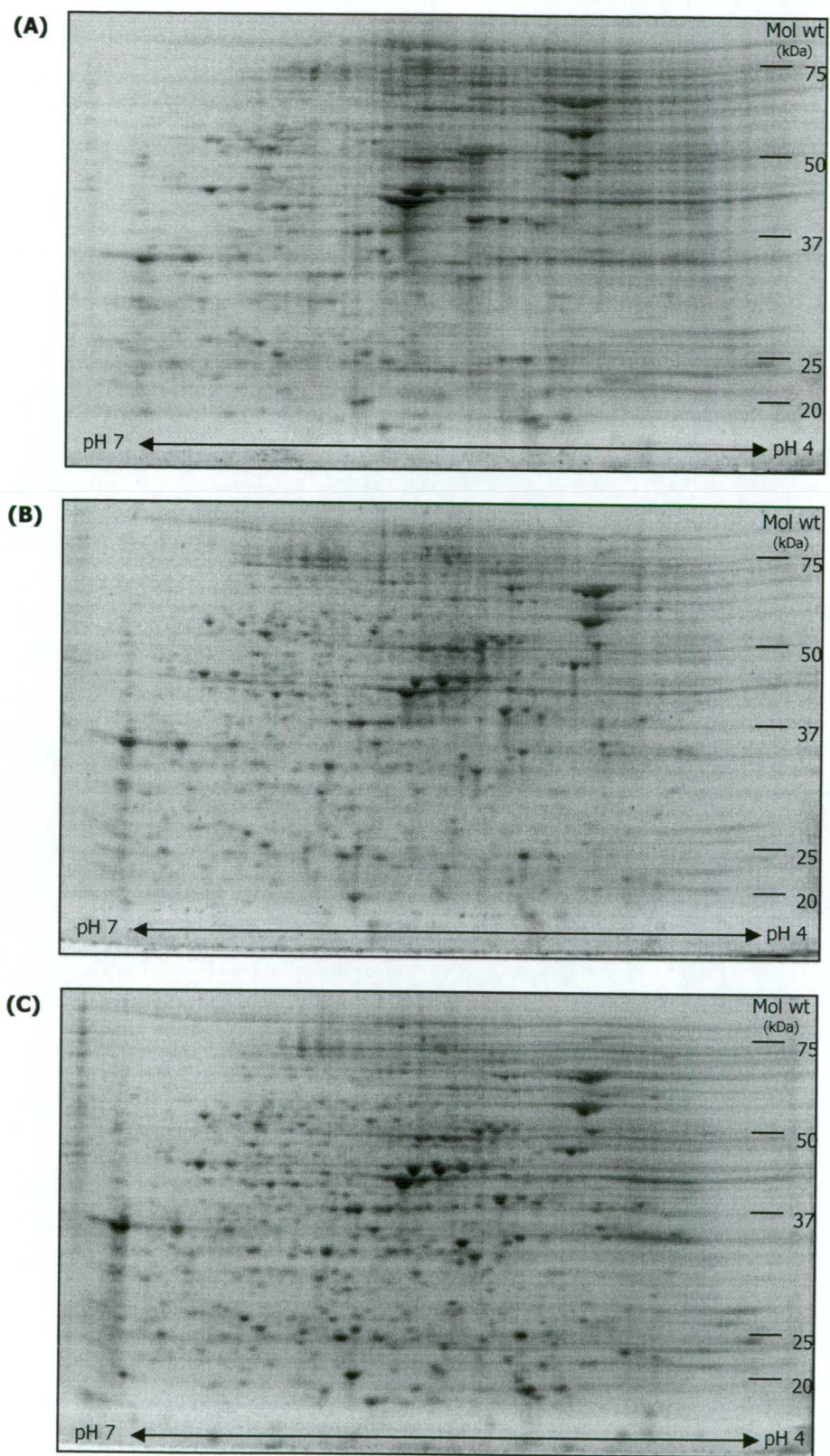
| Temperature<br>(°C) | Time<br>(days) | Water<br>activity | pH   | Inactivation rate <sup>a</sup><br>(log CFU.g <sup>-1</sup> .hr <sup>-1</sup> ) |
|---------------------|----------------|-------------------|------|--|
| 29                  | 3              | 0.916             | 4.85 | 0.0175   |
| 25                  | 4              | 0.872             | 4.81 | 0.00943  |
| 24                  | 4              | 0.962             | 4.68 | 0.00380  |
| 23.5                | 4              | 0.958             | 5.13 | 0.00175  |
| 22.8                | 3              | 0.932             | 6.1  | 0.00612  |
| 23.6                | 2              | 0.952             | 4.57 | 0.00357  |
| 15.1                | 21             | 0.84              | 5.06 | 0.00368  |
| 13.8                | 21             | 0.918             | 4.58 | 0.00289  |
| 28.4                | 3              | 0.914             | 4.88 | 0.0239   |
| 28.4                | 3              | 0.943             | 4.72 | 0.0192   |
| 39.1                | 3              | 0.936             | 4.55 | 0.0516   |
| 32.9                | 4.2            | 0.765             | 5.4  | 0.0296   |
| 32.8                | 3.2            | 0.941             | 4.38 | 0.0197   |
| 33.6                | 3.2            | 0.85              | 4.6  | 0.0297   |
| 32.7                | 3.2            | 0.952             | 4.41 | 0.0201   |
| 23.8                | 5              | 0.954             | 4.6  | 0.00335  |

<sup>a</sup> Overall inactivation rate based on the line of best fit to the data assuming log-linear inactivation.

## **APPENDIX D: Protein Expression Profiles**

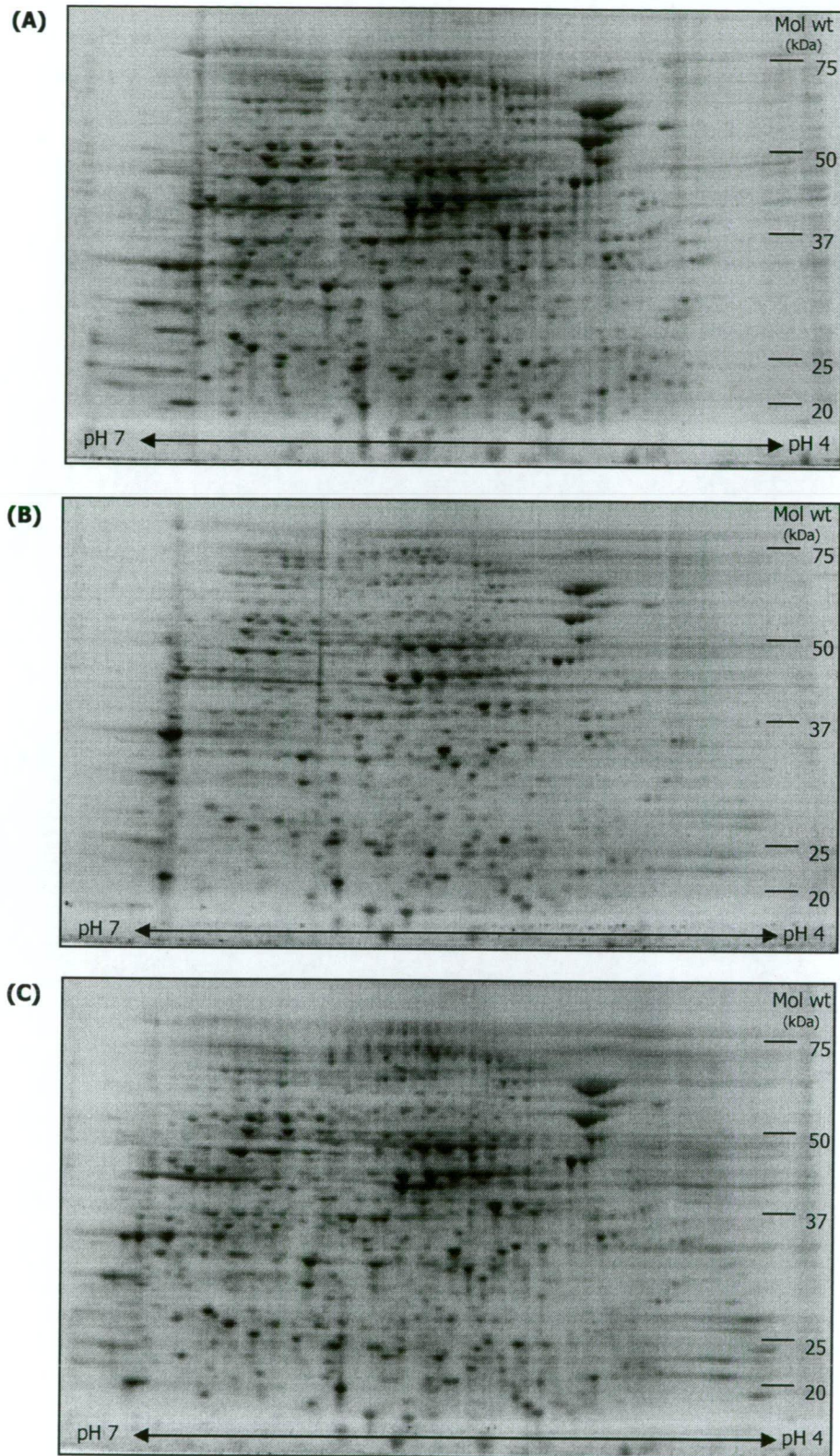
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Figure D.1 – D.3 depict the nine 2-DE gels used to generate the master gel (given in Figure 6.3), to identify proteins by MALDI-TOF MS analyses, and to analyse changes in protein expression between the different treatments as described in Chapter 6. These gels are from a single experiment, which was replicated on two separate occasions.



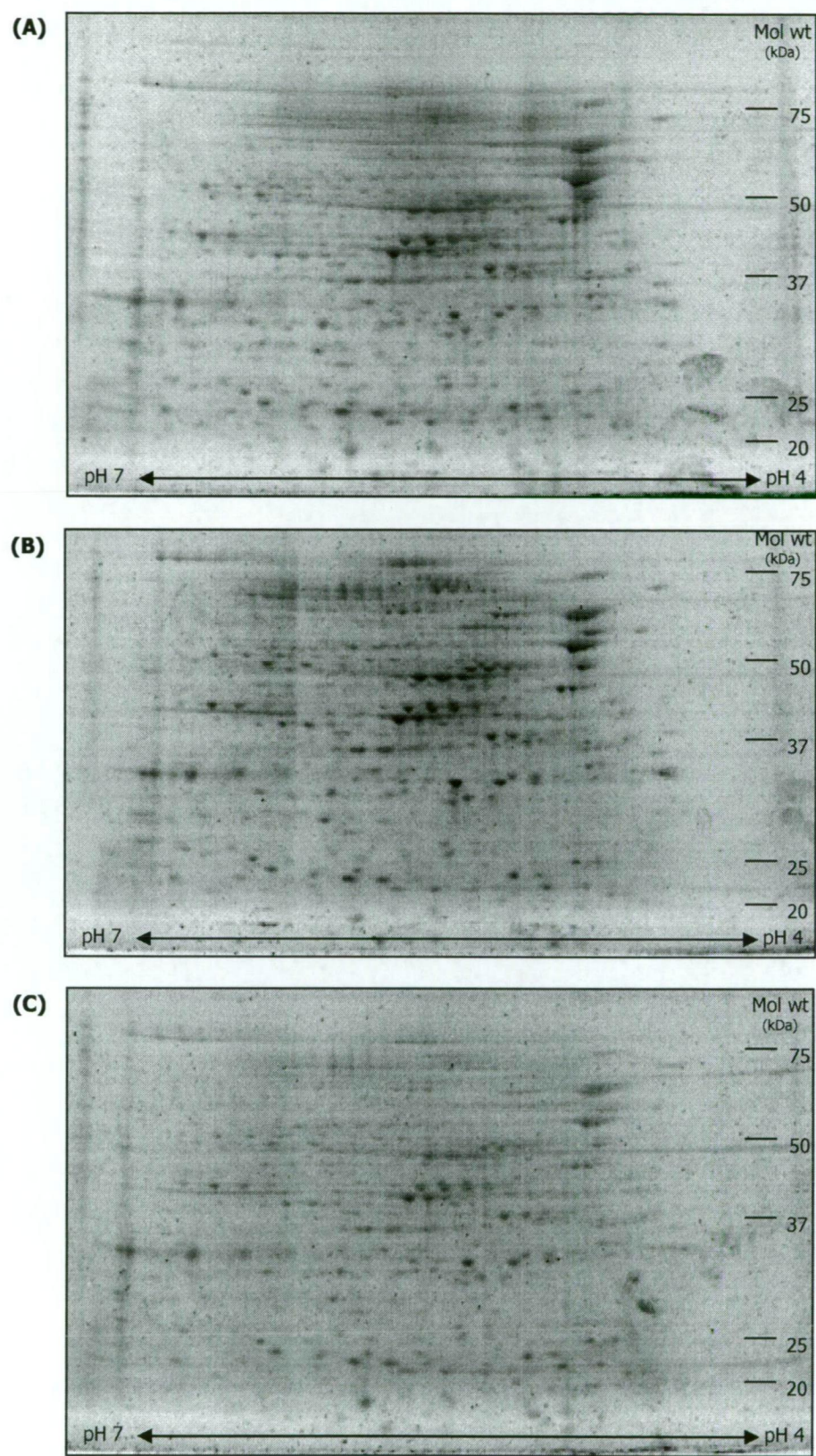
**Figure D.1** Gels showing proteins (100  $\mu$ g total) extracted from the inoculum control populations of *E. coli* M23 (designated A, B and C) and separated by 2-DE in the first dimension (horizontally) according to pI (over a pH range of 4 to 7) and in the second dimension (vertically) based on size. Proteins were visualised by staining with SYPRO ruby. Molecular weight markers were run simultaneously and approximate indications are given on the right.





**Figure D.2** Gels showing proteins (100  $\mu$ g total) extracted from the aged control populations [no  $a_w$  stress, TSB, 25°C, 196.25 hours] of *E. coli* M23 (designated A, B and C) and separated by 2-DE in the first dimension (horizontally) according to pI (over a pH range of 4 to 7) and in the second dimension (vertically) based on size. Proteins were visualised by staining with SYPRO ruby. Molecular weight markers were run simultaneously and approximate indications are given on the right.





**Figure D.3** Gels showing proteins (100 µg total) extracted from osmotically stressed populations [ $a_w$  0.90, TSB, 25°C, 196.25 hours] of *E. coli* M23 (designated A, B and C) and separated by 2-DE in the first dimension (horizontally) according to pI (over a pH range of 4 to 7) and in the second dimension (vertically) based on size. Proteins were visualised by staining with SYPRO ruby. Molecular weight markers were run simultaneously and approximate indications are given on the right.